VOLUME 11 SEPTEMBER, 1940 No. 3

636.2.083.37

261. A STUDY OF THE MORTALITY RATES OF CALVES IN 335 HERDS IN ENGLAND AND WALES (TOGETHER WITH SOME LIMITED OBSERVATIONS FOR SCOTLAND)

BY R. LOVELL
Research Institute in Animal Pathology, Royal Veterinary College, London

AND A. BRADFORD HILL
London School of Hygiene and Tropical Medicine, London

INTRODUCTION

THERE is very little information in this country regarding the magnitude of the losses borne by the dairy industry through the deaths of calves, either pre-natal through abortions and stillbirths, or post-natal through the mortality experienced by the liveborn animals. Some observations made in Scotland suggest that in one area at least such losses may be extremely heavy. Jordan (1), from a survey of twenty-six farms made in Ayrshire in 1931–32, reported that of 784 calves born, as many as 169, or nearly 22 %, died "within a comparatively short time". This high rate of post-natal loss was also recorded by Smith (2), who collected the experiences of fifty-two farmers in the same county during the years 1931–34 and showed an annual death rate of 20 %.

Both authors dealt with heifer calves, as bull calves are mostly disposed of within a few days of birth, and both found a pronounced seasonal swing in the incidence of mortality. In Jordan's observations the calves born during the spring season of 1931 had a death rate of nearly 27 %. For those born in the spring season of 1932 the rate was 24 %, i.e. roughly the same. On the other hand, calves born during the autumn season showed a death rate of only 8 %. Smith's figures were similar, namely, death rates of 25 % in the spring and 8 % in the autumn. In view of these grave losses, their striking seasonal incidence, and the scope for preventive measures which they made obvious, it seemed highly desirable to obtain more extensive data relating to the problem. In particular, it was clearly of value to extend the field of observation to England and Wales to learn whether equally heavy rates of mortality prevailed in its herds.

SCHEME OF INQUIRY

For this purpose a scheme of inquiry was drawn up with the assistance of the Central Council of Milk Recording Societies of England and Wales. The affiliated societies were approached, and through them records were collected

from a large number of farms by the official recorders at their periodical visits to each, at intervals of about 6 weeks.

To make the distribution of herds reported upon representative of the geographical distribution of all herds in England and Wales, the number of the former chosen in each of the Milk Marketing Board regions bore a fixed ratio to the size of the cow population of the region. By this means returns were obtained for 315 herds through forty-three milk-recording societies. In addition further data were collected direct from twenty owners, giving a total of 335 herds. The investigation covered a period of 2 years, from 1 January 1936 to 31 December 1937, but for 46 of the 335 herds the information did not cover the whole period. The geographical distribution of these 335 herds is compared in Table 1 with the corresponding number of cows and heifers in milk or in calf in 1936–7, as published for the Agricultural Divisions of England and Wales (*Ministry of Agriculture and Fisheries, Agricultural Statistics*, 1937, **72**, Part I). It will be seen that the records collected rather overweight the Eastern and West-Midland regions and rather underweight the North-Eastern, Northern and South-Western areas. Combining certain regions to give larger numbers, as is done later in the report for regional comparisons of mortality, it is found that the proportional representation does not differ seriously from the distribution of the cow population. It may be said, therefore, that geographically the herds reported upon are a reasonable though not exact sample of the distribution of the total cow population in England and Wales.

Table 1

Agricultural divisions of England and Wales	Approximate no. of cows and heifers in milk or in calf. Mean of returns for 1936 and 1937	% of total for England and Wales		No. of herds for which data were recorded	% of total for England and Wales	
South-Eastern	289,000	9·4		35	10·4	
Eastern	173,000	5·6	11·1	34	10·1	12·8
North-Eastern	169,000	5·5		9	2·7	
East-Midland	290,000	9·4	21·8	32	9·6	26·6
West-Midland	381,000	12·4		57	17·0	
Northern	304,000	9·9	30·7	19	5·7	28·4
North-Western	636,000	20·8		76	22·7	
South-Western	466,000	15·2		35	10·4	
South Wales	222,000	7·2	27·0	30	9·0	21·8
North Wales	142,000	4·6		8	2·4	
Total for England and Wales	3,072,000	100·0		335	100·0	

In dealing, however, only with milk-recording herds, or with well-known private owners, we have without doubt surveyed a somewhat—perhaps highly—selected class in which a relatively high standard of farm management would probably be the rule. We should, therefore, expect the losses recorded in our inquiry to be minimum figures, which would, on the average, be exceeded on farms falling outside the categories investigated.

From each herd concerned the following data were required:

(*a*) Size and breed of herd.

(*b*) The number of calves born alive, distinguishing sex.

(*c*) The number of calves dying, distinguishing sex and age (a calf was defined as an animal under 6 months of age).

(*d*) The assumed cause of death.

(*e*) The number of stillborn calves.

(*f*) The number of abortions.

During the course of the inquiry it was realized that valuable additional information might also be obtained relating to the methods of calf husbandry adopted by different owners, which might be associated with the mortality rates experienced. Additional forms were, therefore, sent out asking for these details, especially with regard to the feeding of colostrum to the calf, the subsequent methods of feeding, the allowance of water, and whether calves were kept separately or in groups. This information was not secured for all herds, but for what (see later) appeared to be a representative sample of them.

Lastly, for comparative purposes, data relating to the losses experienced in the same years were collected from forty-seven herds of a similar type in Scotland—thirty-four in the Ayrshire district, twelve from the area round Edinburgh and one from Perthshire. This is clearly a very limited sample but one akin to those studied previously by Jordan and Smith.

Results

The total losses reported

Mortality. The basic data resulting from the survey are given in Table 2. From the 335 herds in England and Wales, the average size of which was about forty cattle, there were reported some 25,000 livebirths. Of this total, 1251 liveborn calves, or approximately 1 in 20, died before the age of 6 months. The death rates of females, 5·5 % of those born, is higher than the rate reported for males, 4·4 %, contrary to human experience of infant mortality. The difference is, however, without doubt merely an artefact due to the disposal of bull calves, often about the first or second week of life, so that their births are recorded but not their later mortality. The female death rate is therefore much the better index (see also Table 12 and p. 239). In Scotland the difference between the two sexes is much more pronounced, the death rate of males being only 4·0 %, or approximately the same as in England and Wales, while that of females is 11·4 or twice the English rate. The male rate in Scotland is likely to be still more artificially depressed, as it is the custom in Ayrshire, according to Jordan, to dispose of bull calves within 2 or 3 days (unless kept for stock). Taking, therefore, the female rates as the only reliable index, it seems that the Scottish mortality experience, though considerably below the figures reported by Jordan and Smith, is significantly greater than that which prevails in the English herds of the types reported upon. The latter is only a quarter of the rates reached by Jordan and Smith. These selected

English herds lost, it seems, one female calf before the age of 6 months for each eighteen born.

Table 2. *Total data derived from* (1) *wide survey of England and Wales, and* (2) *limited survey of Scotland*

	England and Wales		Scotland	
No. of herds reported upon	335		47	
No. of cattle in these herds	13212–14547		1730–1774	
Average size of herd*	39–43		37–38	
	M.	F.	M.	F.
No. of livebirths reported	12665	12544	1309	1383
No. of deaths of calves	558	693	52	158
Mortality per 100 livebirths†	*4·4*	*5·5*	*4·0*	*11·4*
No. of stillbirths reported	1231		79	
Stillbirths per 100 total births	*4·7*		*2·9*	
No. of abortions reported	1530		151	
Total pregnancies (live + stillbirths + abortions)	27970		2922	
Abortions per 100 pregnancies	*5·5*		*5·2*	
Total losses (deaths + stillbirths + abortions)	4012		440	
Rate of total loss per 100 pregnancies‡	*14·3*		*15·1*	

* The size of the herd naturally fluctuated over the two-year period and was, therefore, sometimes given as, for example, 45–50. The figures given are the summation of the lower and upper figures.

† The death rate of the males is likely to be materially understated owing to absence of deaths due to the sale of bull calves.

‡ If males had the same death rate as females the total loss would be 14·9 instead of 14·3 per 100 pregnancies in England and Wales and 18·4 instead of 15·1 in Scotland.

Stillbirths. Of the total 26,440 births reported in England and Wales, 1231, or 4·7 %, were stillbirths. (This figure, it may be noted, does not differ appreciably from that reported for the human population of England and Wales, which is annually in the neighbourhood of 4 %.) The limited figures from Scotland give a rather lower rate, 2·9 %, a difference which is unlikely to have arisen by chance with the numbers involved. (At the English rate 129 stillbirths would have been expected in the Scottish herds against the 79 actually observed. Taking the square root of the expectation as the standard error the difference of 50 is over four times its S.E.) Difference in the interpretation of a stillbirth may possibly have played a part, thereby increasing the mortality rate and decreasing the stillbirth rate, but we have no evidence that this occurred.

Abortion rates. The two countries agree closely in their abortion rates. In England and Wales the total number of pregnancies reported was 27,970, and 1530, or 5·5 %, terminated by abortions. In Scotland the corresponding figures were 2922, 151 and 5·2 %. We have no knowledge of the cause of these abortions or what relationship they bear to *Brucella abortus* or other infection.

Rates of total loss. From the number of pregnancies recorded it is possible (with one proviso) to see how many resulted in a full-grown calf. Of the 27,970 pregnancies in England and Wales 1530 resulted in abortions, a further 1231 went to term but resulted in stillbirths, a further 1251 produced living calves which died before reaching the age of 6 months. The total losses were, therefore,

4012 or 14·3 % of the pregnancies. In other words one in seven pregnancies (sufficiently advanced to produce an abortion) failed to produce an adult animal. In Scotland the corresponding loss was 15·1 %, which differs relatively little from the English rate.

It must be noted, however, that these rates are understatements to the extent that the death rates of males are in error due to the selling of bull calves. If it be presumed that their death rate is in reality the same as that of females (from human experience it might be higher, see also Table 12), the English rate of total loss is raised to 14·9 % and the Scottish rate to 18·4. We may justifiably conclude that certainly not less than one pregnancy in seven in England and Wales and not less than one in six in Scotland failed to result in a calf surviving to the age of 6 months.

The rates of separate herds. There was, naturally, much variability in the rates of loss reported for different herds, dependent in part, no doubt, upon the small size of some of the units. The scatter of the rates in England and Wales reported over the two-year period of observation is given in Table 3. (The figures are confined to the 289 herds reporting for the complete 2 years. Their average rates were identical with those given in Table 2 for the total 335 herds.) Approximately half the herds had death rates, stillbirth rates and abortion rates of less than 4 %. At the other extreme nearly one herd in five lost more than 10 % of its liveborn calves, one herd in ten had more than 10 % of its pregnancies end in stillbirths, and one herd in five had more than 10 % end in abortions. The rates of total loss are very variable and the average figure of 14 % given in Table 2 is derived from experiences ranging from no losses at all in the two years to a loss of nearly half the pregnancies reported.

Table 3. *The rates recorded for different herds in England and Wales*

Rate %	No. and proportion of herds with given rates: Death rate of females		Stillbirth rate		Abortion rate		Rate of total loss	
	No.	%	No.	%	No.	%	No.	%
0–	90	*31*	78	*27*	108	*38*	9	*3*
2–	46	*16*	75	*26*	58	*20*	11	*4*
4–	47	*16*	46	*16*	25	*9*	25	*9*
6–	36	*12*	37	*13*	24	*8*	34	*12*
8–	19	*7*	24	*8*	15	*5*	34	*12*
10–	19	*7*	7	*2*	19	*7*	26	*9*
12–	7	*2*	8	*3*	12	*4*	16	*5*
14–	5	*2*	6	*2*	4	*1*	24	*8*
16–	5	*2*	1	—	9	*3*	25	*9*
18–	1		3		2		15	*5*
20–	7		1		3		15	*5*
22–	1		2		2		8	*3*
24–	3				4		12	*4*
26–	—		1	*3*	3		9	
28–	1	*5*			1	*5*	6	
30–	1						6	
32–	1						6	*12*
34–							1	
36–							1	
38 and over							6	
Total	289	*100*	289	*100*	289	*100*	289	*100*

The number of herds in Scotland for which data for the whole 2 years were obtained was only thirty-three, too few for the subdivisions of Table 3. It may, however, be noted that the female mortality rate was over 16 % in one-third of them, while this level prevailed in only 7 % of the herds in England and Wales.

The seasonal incidence of mortality

Both Jordan and Smith, it has been noted, reported a pronounced seasonal swing in the death rates of calves. The method of collecting the present material makes it impossible to examine this factor in a precise manner. The recorders noted the births, deaths and other happenings that had taken place since their last visit, and these events cannot be allocated to exact dates. They have instead been allocated, for the want of a better method, to the quarter in which the visit took place, although it is inevitable that some of the mortality in one quarter may thus be included in the mortality of another. This defect is, however, probably insufficient to account for the smaller seasonal variation in our figures for England and Wales as compared with that reported previously from Scotland. Table 4 shows the rates observed in the 335 herds of England and Wales in the two years 1936 and 1937 and for the forty-seven herds of Scotland in 1937 (for which the quarterly distribution was more exact—no seasonal data were available for 1936).

Table 4. *The seasonal incidence of mortality*

Year and quarter		England and Wales: Death rate % of livebirths		England and Wales: Quarterly rate as % of annual rate		Scotland: Death rate % of livebirths		Scotland: Quarterly rate as % of annual rate	
		M.	F.	M.	F.	M.	F.	M.	F.
1936	1	6·9	7·7	*150*	*133*	—	—	—	—
	2	5·4	5·8	*117*	*100*	—	—	—	—
	3	3·3	4·4	*72*	*76*	—	—	—	—
	4	3·4	5·5	*74*	*95*	—	—	—	—
Total		4·6	5·8	*100*	*100*	—	—	—	—
1937	1	5·1	5·5	*121*	*106*	4·9	10·7	*136*	*123*
	2	3·6	5·6	*86*	*108*	4·0	14·9	*111*	*171*
	3	3·6	4·8	*86*	*92*	1·5	4·0	*42*	*46*
	4	4·3	5·0	*102*	*96*	2·5	3·4	*69*	*39*
Total		4·2	5·2	*100*	*100*	3·6	8·7	*100*	*100*
1936–7	1	5·9	6·6	*134*	*120*	—	—	—	—
	2	4·5	5·7	*102*	*104*	—	—	—	—
	3	3·5	4·6	*80*	*84*	—	—	—	—
	4	3·9	5·3	*89*	*96*	—	—	—	—
Total		4·4	5·5	*100*	*100*	—	—	—	—

Turning first to the Scottish figures, it will be seen that the seasonal changes are of the same order as those reported by Jordan and Smith, although the rates themselves are on a lower level. These authors give female rates of about 25 % in the spring and 8 % in the autumn, a ratio of 3 to 1. The present figures for females are 12·2 % in the first half of the year, and 3·7 % in the second half, a ratio of 3·3 to 1. In the English experience the trend is clearly

in the same direction in each year but on a much lower scale. Taking the combined experience of 1936–7, the rates for the first and second halves of the calendar year are for males 5·2 and 3·7, and for females 6·2 and 5·0. In the first half of the year the male rate is 41 % in excess, and the female rate 24 % in excess, of the corresponding rates in the latter half. (These seasonal changes are more than would be likely to occur by chance. Using the quarterly figures in England and Wales in 1936–7 the departures from a uniform distribution over the year give the following values: males, χ^2 24·30, P less than 0·01; females, χ^2 11·35, P 0·01.)

We may, therefore, conclude that factors inimical to calf life have a greater influence in the first two quarters of the year, chiefly, it seems, in the first quarter, and that—within the limits of this survey—their effect is probably more pronounced in Scotland than in England and Wales. As an interesting corollary it will be seen that the higher death rate of female calves in Scotland, as shown in Table 2, is entirely the product of their high mortality in the first half of the calendar year. In the second half of the year their losses are actually rather less than those observed in England and Wales in the same year.

Whatever these unfavourable factors may be, they do not appear to affect the products of conception *in utero*. The figures of Table 5 show that the

Table 5. *The seasonal incidence of stillbirths and abortions*

Year and quarter	Stillbirths				Abortions			
	England and Wales		Scotland		England and Wales		Scotland	
	Rate % of total births	Quarterly rate as % of annual rate	Rate % of total births	Quarterly rate as % of annual rate	Rate % of total pregnancies	Quarterly rate as % of annual rate	Rate % of total pregnancies	Quarterly rate as % of annual rate
1936 1	4·4	*102*	—	—	4·9	*102*	—	—
2	3·8	*88*	—	—	5·5	*115*	—	—
3	4·3	*100*	—	—	5·1	*106*	—	—
4	4·5	*105*	—	—	4·1	*85*	—	—
Total	4·3	*100*	—	—	4·8	*100*	—	—
1937 1	4·8	*96*	2·4	*71*	6·7	*110*	5·6	*112*
2	4·4	*88*	4·8	*141*	6·1	*100*	3·2	*64*
3	5·4	*108*	4·7	*138*	6·5	*107*	7·1	*142*
4	5·5	*110*	2·9	*85*	5·1	*84*	3·9	*78*
Total	5·0	*100*	3·4	*100*	6·1	*100*	5·0	*100*
1936–7 1	4·6	*98*	—	—	5·9	*107*	—	—
2	4·1	*87*	—	—	5·8	*105*	—	—
3	4·8	*102*	—	—	5·8	*105*	—	—
4	5·0	*106*	—	—	4·6	*84*	—	—
Total	4·7	*100*	—	—	5·5	*100*	—	—

incidence of stillbirths and abortions varies very little by season of the year. In the English figures for 1936–7 combined, there is a suggestion of a slightly higher stillbirth rate in the second half of the year, but the differences are not beyond what might occur by the play of chance ($\chi^2 = 6·73$, P slightly less than 0·1). The fluctuations in the Scottish figures, based upon much smaller numbers, are also insignificant in the technical sense ($\chi^2 = 5·73$, P greater than 0·1). With the abortion rates there is very little variability in England and Wales

over the first nine months of the year, but in both 1936 and 1937 the rate of the final quarter is relatively low. With the volume of data analysed this change is more than would be likely to occur by chance (1936–7, $\chi^2 = 15{\cdot}45$, P less than 0·01), but we can offer no explanation of it. The corresponding fluctuations in the Scottish herds are not more than might occur by chance ($\chi^2 = 6{\cdot}80$, P greater than 0·05).

Seasonal influences, we conclude, are practically confined to post-natal losses. Their origin, it has been suggested, may lie in a lack of vitamin A in the colostrum of the mother in the early months of the year. The cow stores vitamin A while at pasture and generally loses it during the winter, so that the winter pregnancy would be followed by a low vitamin A content of the colostrum in the spring months of the year. As a deficiency of this vitamin is associated with impairment of the local tissue defences to bacteria, Stewart & McCallum(3) studied its relationship to calf mortality, especially that due to white scours or coli-bacillosis. Of a total of 206 calves, 150 were born of cows with more than 250 Blue units of vitamin A per 100 ml. of colostrum. In this group twelve calves (8 %) died from various causes, fourteen (9·3 %) suffered from white scours, one (0·67 %) of which died. Of fifty-six calves whose mothers yielded a lower amount of vitamin A in the colostrum, thirteen (23·2 %) died, fifteen (26·8 %) suffered from white scours, and nine (16 %) died from this disease. The total death rates differ by 15 %, and this has a standard error of 5·1, and so is more than would be attributable to chance. McCandlish(4) considers that factors other than vitamin A may help to explain these seasonal variations. He points out that sunlight during the summer and autumn may provide the autumn calf with more vitamin D than the spring calf can secure, and that a lack of this vitamin may be as important as the lack of vitamin A. The seasonal changes may be associated with the character of the milk curd found in the stomach of the calf, and abnormal curd formation leads to digestive disturbance and even death. Support for this view is obtained from the observation that the milk of stall-fed cows is not as suitable for cheese making as that from cows on pasture.

Regional variations in mortality in England and Wales

To examine the geographical incidence of pre- and post-natal losses in England and Wales, groupings were made of the agricultural divisions as adopted by the Ministry of Agriculture and Fisheries, the grouping being necessary to give sufficient numbers. The incidence of calf mortality in the 289 herds reported for the complete 2 years is shown in Table 6. The average death rates indicate a slight increase in the death rate as one passes northwards, the rates for females being 5·4 and 5·3 % in the South-Eastern and Midland regions and 6·5 and 6·0 in the Eastern and North-Eastern and Northern and North-Western. The male rates, though certainly much less accurate than the female, have the same trend. The South-Western and Wales area is discrepant, having the highest male but the lowest female death rate. The distribution of

rates given at the bottom of the table shows that there is certainly no great variation in mortality by region in the type of herd for which records were collected. In each area approximately half the herds enjoyed a relatively low rate of mortality (under 4 %) and, with the exception of the Midlands, 10–12 % had relatively high rates (12 % or more). A suggestion of a higher rate in the northern counties is in keeping with the higher rates observed in Scotland, but we regard it as no more than a suggestion.

Table 6. *The post-natal mortality rates in different regions of England and Wales in* 1936–7

	South-Eastern	Eastern and North-Eastern	East- and West-Midland	Northern and North-Western	South-Western and Wales
No. of herds	32	36	69	85	67
Male births	2163	1526	3149	2951	1786
Female births	2100	1537	3021	3052	1818
Male deaths	75	72	118	137	107
Female deaths	113	100	160	184	79
Death rate % M.	3·5	4·7	3·7	4·6	6·0
F.	5·4 } 4·4	6·5 } 5·6	5·3 } 4·5	6·0 } 5·3	4·3 } 5·2
Death rate %	% of herds with given rate*				
0–	59	51	53	49	50
4–	25	24	27	29	22
8–	5	15	14	10	16
12 and over	11	10	6	12	12

* The male and female death rates of each herd were used separately in these calculations.

Figures for the pre-natal losses in different regions are given in Table 7. The average stillbirth rate varies negligibly with the exception of a rather low rate in the Eastern and North-Eastern area. The abortion rate fluctuates more widely, being as high as 7·3 % in the Northern and North-Western and as low as 3·9 % in the South-Western and Wales. With the relatively small

Table 7. *The pre-natal rates of loss in different regions of England and Wales in* 1936–7

	South-Eastern	Eastern and North-Eastern	East- and West-Midland	Northern and North-Western	South-Western and Wales
No. of stillbirths	247	120	309	300	172
Stillbirth rate %	5·4	3·8	4·8	4·8	4·6
Stillbirth rate	% of herds with given rate				
0–	47	61	51	54	52
4–	22	31	30	28	30
8–	16	8	9	11	12
12 and over	16	—	10	7	6
No. of abortions	236	139	397	496	152
Abortion rate %	5·3	4·2	5·8	7·3	3·9
Abortion rate	% of herds with given rate				
0–	56	69	52	47	70
4–	13	14	22	19	13
8–	19	8	10	14	9
12 and over	13	8	16	20	8

numbers involved, these average rates may, however, be rather heavily affected by the unfavourable experience of one or two herds. The distribution of the individual herd rates shows that all regions experience high, medium and low stillbirth and abortion rates and to roughly the same extent. From a statistical test applied to the numbers falling in these categories in each region, we conclude that the observed variability is no more than might occur by chance (stillbirth rates, $\chi^2 = 9{\cdot}1$, $n = 12$, P 0·7, abortion rates $\chi^2 = 14{\cdot}82$, $n = 12$, P about 0·25).

With the exception of a slightly higher rate in the Northern and North-Western region, the rates of total loss (mortality + stillbirths + abortions) are very similar, viz. South-Eastern 14·3 %, Eastern and North-Eastern 13·0, Midland 14·3, Northern and North-Western 16·4 and South-Western and Wales 13·0. Within the selected group of herds reported it is clear that geographical location plays little, if any, part in the total and different kinds of losses experienced.

Losses in relation to size of herd

From an analysis by size of herd it appears that the losses experienced are not greatly influenced by this factor (Table 8). With the post-natal death rate the males and females both indicate a rather higher rate of loss in the smallest herds of all; thereafter there is relatively little change. The sex rates differ very little from one another in the small herds, and it may be that there is less selling of bull calves in this group. As usual, attention is, therefore, better given to the rates for females. The average female death rate in herds of under twenty is 6·7 % and in herds of twenty and over it is 5·4, the former being 24 % in excess of the latter. Examination of the individual herd rate gives the following results: in herds numbering under twenty the female death rate was low (under 4 %) in 46 %, medium (4–12 %) in 28 %, and high (12 % or more) in 26 %. The corresponding percentages for herds of twenty or more were 47, 44 and 9. There is clearly a tendency for high rates to prevail more frequently in the smaller herds, the difference in the distributions being significant ($\chi^2 = 10{\cdot}7$, P less than 0·01). On the other hand, very low rates are certainly not a prerogative of large herds, for both groups have an equal proportion enjoying losses of under 4 %.

Table 8. *Pre- and post-natal losses in relation to size of herd. Average rates for England and Wales*

Size of herd*	No. of herds	Births		Deaths		Still-births	Abortions	Death rate			Still-birth rate	Abortion rate	Total loss rate
		M.	F.	M.	F.			M.	F.	M. + F.			
Under 20	39	632	653	41	44	72	60	6·5	6·7	6·6	5·3	4·2	15·3
20–39	131	3761	3778	161	187	345	380	4·3	5·0	4·6	4·4	4·6	13·0
40–59	72	3166	3246	146	175	332	493	4·6	5·4	5·0	4·9	6·8	15·8
60 and over	47	4016	3851	159	230	399	487	4·0	6·0	4·9	4·8	5·6	14·6

* Where the herd size was given in the form "36–42" the mean of the two values was used in allocating it to its group.

The stillbirth rate shows no uniform change with size of herd, though the highest rate is again found in those of the smallest size. With the numbers involved the difference is of only doubtful significance.

With the abortion rate there is a suggestion of a rise with increasing size of herd. The distribution of herd rates was as follows:

Abortion rate	Size of herd			
	Under 20	20–39	40–59	60 and over
	% of herds having given rates			
0–	67	63	52	43
4–	26	25	30	41
12 or over	8	13	18	15

There is a progressive decline in the number of herds having low rates as the size of the herd increases, and a corresponding rise in the number with higher rates. The changes are, however, of doubtful significance ($\chi^2 = 8{\cdot}7$, $n = 6$, P nearly 0·2).

The result of these changes in the different rates with size of herd is that the rate of total loss shows no uniform upward or downward trend. We conclude, therefore, that the smallest herds suffer a somewhat higher rate of calf mortality, that possibly losses by abortion may increase with increasing numbers in the herd, but that the rate of total loss is likely to be, on the average, much the same in this class of herd whatever its size.

Methods of calf husbandry

In exploring possible relationships between the rates of mortality and the methods of husbandry employed, we were limited to 240 herds in England and Wales (out of the total of 335) from which such information was obtained. Comparison of the average rates for the total group and for this narrower sample does not suggest that they were in any way a selected group. The figures were as follows:

	Death rate of calves (both sexes)	Stillbirth rate	Abortion rate	Rate of total loss
All 335 herds	5·0	4·7	5·5	14·3
240 herds recording throughout 2 years and giving details of husbandry	5·0	4·7	5·6	14·5

Colostrum. In 170 of the 240 herds (71 %) the calf was allowed to take the colostrum from the mother, in 65 (27 %) it was given it by bucket, and in only 5 (2 %) was no colostrum given. The last group is, unfortunately, too small to be very informative. Of herds in which colostrum was taken from the mother ten were of size 60–99 cows, while none happened to be of this size in the group having colostrum by bucket. The remainder had a similar size distribution. Excluding these ten, to keep constant the possible effect of size of herd on mortality, the death rates given in Table 9 were reached. Although the small size of the group given no colostrum must be remembered, it will be seen that the calf mortality rate rises for both sexes as one passes from the

group allowed colostrum naturally to those given it by bucket and finally to those from which it was withheld. Those having colostrum by bucket had a mortality rate some 20 % in excess, those with no colostrum 30–40 % in excess, of the rates in calves given colostrum by the mother. (With the two larger groups the difference is not statistically significant with the males, $\chi^2 = 2{\cdot}0$, P 0·16, but significant with the females, $\chi^2 = 4{\cdot}4$, P 0·036.)

Table 9. *Relation of colostrum to mortality*

Group	Recorded births		Recorded deaths		Death rates %		Relative rates	
	M.	F.	M.	F.	M.	F.	M.	F.
160 herds having colostrum from mother	5694	5789	240	284	4·2	4·9	100	100
65 herds having colostrum from bucket	1926	1960	96	120	5·0	6·1	119	124
5 herds having no colostrum	219	189	12	13	5·5	6·9	131	141

It was thought possible that geographical variations in husbandry might play a part in these differences. As a test thirty-five of the herds given colostrum by bucket were paired with an equal number given colostrum by the mother, region being equalized and size of herd being made as similar as possible (the remaining thirty could not be paired precisely). As the figures of Table 9 showed that the magnitude of the difference between the two groups in their rate of mortality was closely alike in the two sexes, males and females were taken together in spite of the defects in the male figures, to give a larger basis to the present comparison. In the thirty-five herds given colostrum by the mother there were 2529 live births and 124 deaths, a death rate of 4·9 %. In the thirty-five herds given colostrum by bucket, there were 2471 livebirths and 143 deaths, a death rate of 5·8 %. This is 18 % in excess of the former rate, and differs little from the excesses shown in Table 9.

Lastly we may note that taking the death rates experienced by males and females separately in the individual herds, 68 % of these rates were below 6 deaths per 100 livebirths in herds given colostrum by the mother against 61 % in herds given colostrum by bucket and 50 % in herds given no colostrum (based upon ten rates only). Correspondingly, 17 % of the rates in the first group were as high as 9 deaths or more per 100 livebirths against 23 % in the second group and 30 % in the third.

Although the differences are not large, we think that there is evidence that the giving of colostrum naturally is of some benefit and likely, on the average, to reduce the rate of calf mortality. This conclusion is in keeping with observations reported by Smith & Little (5). These authors pointed out parallel series of observations which could be linked, namely, the high mortality of calves in the first week of life and the transference of immune bodies from mother to foetus or nurslings. It is recognized that the relative importance of placental and colostral transmission of immunity varies in the different animal species. In bovines the colostrum is of major importance, and Smith & Little indicated that the colostrum of the mother played an important role in the prevention of white scours which attacks newborn calves. All of ten calves which were

allowed to take colostrum after birth survived, whereas eight of twelve which were deprived of it died and one was killed moribund. In the few herds in our experience in which colostrum was withheld, nothing like this excessive mortality prevailed. In a later paper (Smith (6)) it was shown that the colostrum of normal cows usually contains antibodies to strains of *Bact. coli* of the "scours type". It was assumed that the cows became immunized to the flora prevailing in the herd and this supported the earlier work (Smith & Little (7)) that, as serum was an efficient substitute, the effect of colostrum was due to the transfer of antibodies. As has been noted, when dealing with the seasonal incidence of mortality, Stewart & McCallum (3) stress their belief in the importance of the vitamin A content of the colostrum of the mother in the prevention of infection in the early days of the calf's life. This seasonal trend in mortality may add some support to the importance of vitamin A as there is an accumulation of this factor in the liver whilst cows are at pasture. On the other hand, it must be remembered that dietary and other factors may influence the production of antibodies, and this would tend towards a seasonal variation. For example, Anderson, Fraser & Mackie (8) found that the natural bactericidal reaction of sheep serum towards a strain of *Bact. coli* was higher in grazing animals than in comparable sheep on an artificial ration. Whatever the virtue of colostrum, it is obviously of importance to the calf in its early life.

Methods of feeding. There is a general consensus that suckled calves do better than bucket-fed calves (McCandlish (4)), but this method may not be economical on some dairy farms. Sheehy (9) and Hewison (10) were of opinion that the latter method of feeding may contribute to loss of life. Sheehy found that the digestive disturbance which is common in calves so reared is due to abnormal curd formation in the fourth stomach, and that this was often a cause of death. Hewison stresses that the large quantities of milk given by the bucket in the early days may upset the calf's stomach, and that much trouble and sometimes total loss is incurred by overfeeding in the first fortnight of life. This form of feeding may mean that the calf takes at one meal as much as it would obtain naturally from the cow over a much longer period of time.

It was the practice in the majority of the herds in the present survey for the calf to be fed by the bucket. In some cases the milk was diluted with water. In only 26 herds (11 %) was the calf allowed to suckle the mother, in 159 (66 %) it was fed by bucket with undiluted milk and in 55 (23 %) it was fed by bucket with diluted milk. (This excludes three herds in which a dried milk was apparently used, and includes three entered twice, in which males and females were fed by different methods.) Regional variations in practice were apparent and Table 10, therefore, shows the mortality experienced in different areas (combining males and females). It is clear that there is no appreciable difference between the mortality rates of the different feeding groups in the South-Eastern, Eastern and North-Eastern and Midland regions. With the relatively small numbers involved in some groups the equality of the rates is striking.

In the Northern and North-Western and South-Western and Wales regions there is much more variability, but the rates are based upon so small a number of births in three of the six groups that we are not inclined to stress these differences.

Table 10. *Relation of mortality to method of feeding. Average rates by regions*

Region	Calves suckled		Calves bucket-fed undiluted		Calves bucket-fed diluted	
	No. of births (M., F.)	Death rate %	No. of births (M., F.)	Death rate %	No. of births (M., F.)	Death rate %
South-Eastern	1318	4·2	2148	4·1	395	4·1
Eastern and North-Eastern	—	—	2172	5·8	482	5·8
Midland	603	3·6	2491	3·9	818	3·9
Northern and North-Western	162	9·3	3023	4·9	1573	6·8
South-Western and Wales	525	6·1	1946	4·7	660	7·0
All areas	2608	4·8	11780	4·7	3928	5·8

Further, taking the country as a whole, we find a very similar distribution of herd rates in the different feeding groups (Table 11). There is clearly no difference here between the herds in which the calves were suckled and those in which they were fed with undiluted milk by bucket. Relatively high and low rates are observed with approximately equal frequency. There is a slight excess of the higher rates in the herds in which the calves were fed by bucket with diluted milk—27 % of them have rates of over 9 compared with 18 and 16 % in the other two groups. This difference is, however, not more than might easily occur by chance (χ^2 of (1)+(2) against (3)=7·3, P greater than 0·1). Our figures indicate, we think, that mortality in these herds was not differentially influenced by these three methods of feeding.

Table 11. *Relation of mortality to method of feeding. Distribution of herd rates (male and female rates calculated separately)*

Death rate %	Calves suckled (1)	Calves bucket-fed undiluted (2)	Calves bucket-fed diluted (3)
	% of herd rates falling at different levels		
0–	42·9	45·9	42·2
3–	24·5	22·2	16·5
6–	14·3	15·8	13·8
9–	10·2	9·5	16·5
14 or more	8·2	6·6	11·0
No. of rates upon which percentages are based	49	316	109

Water. In 161 herds (67 %) the calves were allowed to drink water, and in 79 (33 %) they were not allowed to do so. The death rates in the two groups differ negligibly, being as follows:

	Death rate	
Water allowed	M. 4·3	F. 5·5
Water not allowed	M. 4·5	F. 5·8

In the group not allowed water the average death rate was a fraction higher in each sex. There was, however, some regional variation in allowing or not

allowing water. We therefore calculated the regional rates separately for the larger group of herds in which water was given. We then applied these rates to the livebirths recorded in the same regions in the herds in which water was not given. At such regional rates the calves not allowed water would have experienced 126 male deaths and 156 female deaths. The deaths actually recorded were 125 and 167. The differences are again negligible, and we conclude that the giving or withholding of water had no effect upon the mortality rate.

Method of keeping calves. Attempts were made to find out if the practice of keeping calves separately or in groups has any effect on the mortality. Another inquiry concerned the position of the calf pens, as to whether they were in the open or under cover. In only 38 of the 240 herds (16 %) were the calves kept separate. In these herds there were 1394 male and 1434 female livebirths and 72 male and 102 female deaths. If the calves had died in each region at the regional rates experienced by the calves in herds in which they were kept together, the deaths would have been 65 males and 82 females. The relatively large discrepancy between the observed and expected deaths of females (102 to 82) is due entirely to an unusually high death rate in one large herd. If this be excluded the observed deaths of calves kept separate are 56 males and 71 females and the expected death rates at the rates of calves kept together are 59 and 75, clearly insignificant differences on the figures available.

Only nine owners reported their calf pens in the open. In these herds there were 297 male and 292 female livebirths and 11 male and 15 female deaths. At the regional rates of the remainder the expected deaths are 12 and 15. There is, therefore, no evidence in our figures that these methods of calf keeping influence mortality.

Age of calves at death.

With most calves that died information was supplied as to the age at death. From the method of collecting the data—by visits to farms at approximately 6-weekly intervals—these particulars cannot, we realize, be strictly accurate, but we believe them capable of giving a broad picture of the actual events. The resulting figures are shown in Table 12. The first point of interest is the difference between the male and female mortality rates. In the first week of life the death rate of males was 3·05 %, while that of females was 2·42. At this early stage of life the rate of loss of males was 26 % above the rate of loss of females. This difference very closely parallels, it may be noted, human experience. In the three years 1936–8, the male infant mortality rate in England and Wales was 28 % above the female rate in the first week of life. In the human population this male excess remains at much the same level for the rest of the first year of life. On the other hand, in our calf population, the male rate falls far below the female rate at the subsequent ages. This feature is, we believe, as already pointed out, merely an artefact due

to the disposal of bull calves, and the age distribution of their deaths strengthens our view that the male death rates must be regarded with considerable reserve throughout this report.

Turning to the female deaths, it will be seen that very nearly half of them were stated to have taken place in the first week of life, 61 % within the first two weeks and rather more than three-quarters in the first month. As might be expected, the early stages of life are the critical periods, and this is in conformity with the experience of other authors. Mejlbo(11) states that of 5042 calves, which were submitted for post-mortem examination, the ages varied from a few hours to 1 year—the majority being 2–10 days old. Of the eight calves dying in the experiment recorded by Smith & Little(5), seven were less than 7 days old and the other was 11 days. Of the 100 calves examined bacteriologically by Lovell & Hughes(12), twenty-five were less than 7 days old and twenty-eight were from 7 to 14 days of age. It is possible that there was in this record an excess of deaths in the second week of life compared with the first week because of the inclusion of twenty-eight calves which had been bought for a nutritional experiment. Of the 100 calves examined, thirty-seven died from coli-bacillosis, twenty-two by the 7th day and a further eleven by the 14th day. It is thus clear that an important hazard of the first week or fortnight of the calf's life is white scours of coli-bacillosis. The distribution of ages in our series would support this. Although the original forms sent out asked for the cause of death it was obvious that no great reliance could be placed on these records. A large number of young calves were, however, recorded as having died from white scours and in many of these the diagnosis was probably correct in view of the widespread incidence of the condition and the ages at which the deaths occurred.

Table 12. *Calf mortality rates at different ages (approximate figures)*

	No. of deaths recorded		Death rate % of total livebirths		Male death rate as % of female death rate	Deaths of females as % of deaths at all ages
Age in weeks	M.	F.	M.	F.		
Age unknown	31	16	—	—	—	—
0– 1	386	304	3·05	2·42	126	45
1– 2	49	110	0·39	0·88	44	16
2– 3	25	58	0·20	0·46	43	9
3– 4	25	45	0·20	0·36	56	7
4– 8	25	78	0·20 (0·05)*	0·62 (0·16)*	32	11
8–12	8	45	0·06 (0·02)	0·36 (0·09)	17	7
12–26	9	37	0·07 (0·02)	0·29 (0·07)	24	5

* Average weekly rates in interval.

Summary

1. With the assistance of the Central Council of Milk Recording Societies of England and Wales and its affiliated Societies, particulars were obtained of the calf losses, pre-natal and post-natal, sustained in 1936–7 by 315 herds situated in different counties of England and Wales. Further data were

collected from twenty private owners in England and from forty-seven herds in a limited area in Scotland.

2. The herds in England and Wales were chosen in such a way as to give a fair representation of the geographical distribution of the cow population throughout the country. It is obvious, however, that they are a selected sample in which a relatively high standard of husbandry might be expected to prevail and their rates of mortality, therefore, will probably be below that of the general run of herds.

3. Owing to the early disposal of bull calves the losses of males after the first week of life are likely to be considerably understated. It is, therefore, wise to confine attention mainly to the figures for females.

4. In the 335 herds of England and Wales 12,544 female calves were born and of these 693, or 5·5 %, died before the age of 6 months.

5. Of 26,440 total births reported in England and Wales, 1231, or 4·4 %, were of stillborn animals.

6. 1530 abortions were reported or 5·5 % of the total pregnancies recorded.

7. Adding together the livebirths, stillbirths and abortions, a total of 27,970 pregnancies was recorded and of these 14·3 %, or 1 in 7, failed to produce a calf surviving to the age of 6 months. (This figure must be an understatement owing to the absence of male deaths previously referred to.)

8. Mortality in England and Wales shows some seasonal swing, being higher in the first half than in the second half of the year. This variation is less than that observed in Scotland but the method of collecting the data in England and Wales may have artificially reduced the real seasonal changes. Stillbirths and abortions were little, if at all, influenced by seasonal factors.

9. Within the selected group of herds reported upon, geographical location in England and Wales plays little part in the losses sustained. There is probably a slight increase in the death rate as one passes northwards, and in Scotland it was significantly higher than in England and Wales.

10. The smallest herds (under twenty cows) suffered the highest rate of mortality, but generally the differences between different sizes of herd were not marked.

11. Relation of the observed calf mortality rates to the methods of calf husbandry reported suggested (*a*) that there is an advantage in allowing the calf to take the colostrum naturally from its mother, a conclusion in keeping with the experimental evidence; (*b*) that the mortality in these herds was not influenced by the three methods of feeding (suckling, bucket fed with undiluted milk, and bucket fed with diluted milk); (*c*) that mortality was not influenced by the giving or withholding of water to drink.

12. Nearly half the deaths of female calves took place in the first week of life and three-quarters in the first month (based upon approximate figures). Early post-natal life is clearly the most critical period. No reliable figures regarding causes of death were collected, but it is probable that coli-bacillosis or white scours is an important component.

ACKNOWLEDGEMENTS

This survey was made possible by a grant recommended by the Agricultural Research Council. We are grateful to all those who helped us in different ways to collect the data and especially to the Central Council of Milk Recording Societies (Secretary: D. J. Rumbold, Esq.), to Dr J. Wilkie, of the Hannah Dairy Research Institute, to Mr C. B. Davidson, M.R.C.V.S. and to Dr F. C. Minett.

REFERENCES

(1) JORDAN, L. (1933). *Vet. J.* **89**, 202.
(2) SMITH, R. M. (1934). *Vec. Rec.* **14**, 1004.
(3) STEWART, J. & MCCALLUM, J. W. (1938). *J. comp. Path.* **51**, 290.
(4) MCCANDLISH, A. C. (1939). *Res. Bull. W. Scot. agric. Coll.* **7**.
(5) SMITH, T. & LITTLE, R. B. (1922). *J. exp. Med.* **36**, 181.
(6) SMITH, T. (1930). *J. exp. Med.* **51**, 483.
(7) SMITH, T. & LITTLE, R. B. (1922). *J. exp. Med.* **36**, 453.
(8) ANDERSON, E. J. M., FRASER, A. H. H. & MACKIE, T. J. (1935). *J. comp. Path.* **48**, 51.
(9) SHEEHY, E. T. (1934). *Sci. Proc. R. Dublin Soc.* **21**, 73.
(10) HEWISON, H. V. (1939). *J. Minist. Agric.* **46**, 18.
(11) MEJLBO, E. (1934). *Acta path. micro. Scand.* **18**, 143.
(12) LOVELL, R. & HUGHES, D. L. (1935). *J. comp. Path.* **48**, 267.

(*MS. received for publication* 1 *June* 1940)

631.593.5:576.8

262. THE MICROBIOLOGY OF SILAGE MADE BY THE ADDITION OF MINERAL ACIDS TO CROPS RICH IN PROTEIN

II. THE MICROFLORA

By A. CUNNINGHAM and A. M. SMITH

From The College of Agriculture, Edinburgh

In Part I of this paper(4) it was shown that, although the A.I.V. method for preserving green fodder is capable of maintaining in a satisfactory state of preservation even highly nitrogenous materials such as pure leguminous crops, it does not completely inhibit the growth of bacteria and other micro-organisms. It is obvious, however, that owing to the high acidity, the growth conditions prevailing in silage of this type are of an unusual nature. It is true that in silage made by the low-temperature fermentation process the *p*H is not infrequently reduced to values under 4·0. Such figures, however, are reached gradually as a result of the activity of a microflora which has started growth at much higher levels. The micro-organisms which grow in A.I.V. silage, on the other hand, must be capable of initiating growth at *p*H values below 4·0. It is probably always more difficult to begin growth at low *p*H values than to reduce the *p*H slowly from a higher figure to the same low level. There are, therefore, grounds for anticipating that a study of the microflora of samples of A.I.V. silage may disclose the presence of unusual types of micro-organisms.

A perusal of the literature indicates that little is known about the types of organisms which are capable of surviving and multiplying in silage made by the addition of mineral acids to crops rich in protein. There are a few general statements which have been summarized on p. 402 of Part I of this paper(4), but no detailed investigation of silage of this type appears to have been made. A study of the microflora of A.I.V. silage seemed therefore to be warranted.

For this part of the investigation a selection was made of organisms representative of the main types referred to in Part I. Special attention was paid to the lactobacilli, streptococci, micrococci and sarcinae from which groups some seventy strains were reserved for detailed study. In making this selection, as a rule only those strains were included which had been obtained from samples having a mean *p*H value between 3·0 and 4·0: an exception was made in the case of one type (III) which was much more numerous in samples with *p*H values above 4·0 than in those having *p*H values below this figure. A less complete examination was made of strains of yeasts and of

gas-producing bacteria isolated from certain of the samples of silage. All strains for detailed study were first purified by plating at least twice on dextrose agar.

Methods

In the case of the true lactic acid bacteria, the characteristics which were studied are referred to below. They included many of the common morphological and cultural characteristics but, as is usual in such investigations, most of these proved to be of limited value in the classification of the organisms. Prior to the study of the biochemical characteristics, an investigation of various sources of nitrogen was made, and the medium selected for the biochemical work was based on the results obtained. A preliminary grouping of the organisms was made on their ability to produce catalase and reduce nitrate, their action on aesculin and their fermentation reactions. Selected strains from the various groups were then employed in further biochemical work, mainly of a quantitative nature; the characteristics studied in this part of the investigation were the quantity and form of lactic acid produced and the amounts of volatile acid and carbon dioxide formed from dextrose. The determination of carbon dioxide was finally extended to all strains. As a rule, media were inoculated with loopfuls of young cultures in dextrose bouillon.

The characteristics studied were:

(1) *Form, size, arrangement and Gram-staining reaction.* These were determined in films prepared from young dextrose agar slope cultures and stained by Kopeloff & Cohen's(14) modification of Gram's method. The staining reactions of representative strains were checked by the aniline gentian violet—Lugol's solution—alcohol technique.

(2) *Ability to form spores.* Old nutrient agar slope cultures stained by dilute fuchsin and by the nigrosine spore staining technique ((3), p. 59) were examined. Inocula from nutrient agar slope cultures, incubated for 14 days at their optimum temperature, were transferred to tubes of dextrose bouillon and heated at 80° C. for 10 min.; they were then incubated at suitable temperatures in order to determine whether they were capable of producing growth.

(3) *Motility.* Hanging-drop preparations from young dextrose bouillon cultures were examined. In certain cases also the semi-solid agar method (0·3%) was used, but it was found to yield indefinite and inconsistent results. A few transfers in the semi-solid medium sometimes proved valuable, however, in stimulating the motility of sluggish strains. Young dextrose agar slope cultures representative of types which showed motility were also stained by means of Gray's(10) flagella stain.

(4) *Colonies on dextrose agar.* During a period of about a fortnight, colonies on dextrose agar plates incubated at 22° C. were examined at intervals by means of an ocular-objective combination magnifying about 100 times. The colonies of the majority of the strains were similar in appearance; they varied, however, in size. The longest linear dimension of the deep colonies was measured. Deep colonies were selected for measurement because they are less liable to be influenced by variable factors than surface colonies or those between the medium and glass.

(5) *Liquefaction of gelatin.* Stab cultures in dextrose gelatin incubated at 22° C. were examined at intervals during a period of several months.

(6) *Growth in milk media.* Cultures in litmus milk, 1% dextrose litmus milk and 0·2% Difco yeast extract litmus milk and in 1 in 200,000 methylene blue milk, all incubated at 22° C., were observed at intervals for 2 months; acidification, curdling and reduction were noted.

(7) *Formation of slime from saccharose.* Strains were cultivated on 1% saccharose agar slopes incubated at 22° C. The streptococci were also transferred to 1% molasses agar slopes at 30° C. Neither of these media yielded positive results with any of the strains examined. Satisfactory differentiation of slime-forming from non-slime-forming strains was, however, obtained by cultivation in 5% saccharose gelatin stabs at 22° C.

(8) *Utilization of yeast preparations, casein digests and nutrient bouillon as sources of nitrogen.* The sources of nitrogen tested were:

AUTOLYSED YEAST—prepared as described by Cunningham ((3), p. 39) but diluted to three times its volume with distilled water.

YEAST EXTRACT—prepared from baker's yeast by the method described by Fred *et al.*(9).

DIFCO YEAST EXTRACT—1%.

PEPTIC CASEIN DIGEST—prepared as described elsewhere ((3), p. 31) and diluted to twice its volume with distilled water. The 10-day digest was compared with a preparation digested for 63 days but no appreciable difference was observed.

DIFCO TRYPTONE—1%.

NUTRIENT BOUILLON.

To each medium, 0·5% of dextrose was added and the medium was tubed in 5 ml. quantities and sterilized. The cultures were incubated at 30° C. After periods of 1–2 days they were plated quantitatively on dextrose agar incubated at 30° C. It should be stated that the yeast extract contained small quantities of fermentable compounds but the influence of these was considered to be insignificant when compared with that of the added dextrose.

The results of these tests will be referred to in detail at a later stage. At this point it may suffice to state that autolysed yeast was almost invariably the best source of nitrogen and that, in almost all cases, peptic casein digest was unsatisfactory. The relative values of the others varied with the type of organism tested. For fermentation and other tests the basal medium selected was nutrient bouillon+1% Difco yeast extract+1% Difco tryptone; this was used either undiluted (2 Y.T.B.) or diluted to half concentration (Y.T.B.). In an attempt to obtain a better basal medium for the types of organisms which grew poorly, infusion bouillon, yeastrel and peptic and tryptic digests of glutenin were tested but none of these media gave better results than Y.T.B.

(9) *Optimum temperature for growth.* Medium: Y.T.B.+2% dextrose, sterilized together. Duplicate tubes (5 ml.) were incubated for 14 days at 22–23, 30 and 37° C. and titrated with 0·1 *N* sodium hydroxide.

(10) *Lower limiting pH for growth.* Medium: Y.T.B.+2% dextrose, tubed in 20 ml. quantities and sterilized and adjusted to the following *p*H values (determined by means of a quinhydrone electrode) by additions of sterile diluted hydrochloric acid: 6·70, 4·77, 4·11, 3·79, 3·50 and 3·13. The media were incubated for 2–3 days at 30° C. to check their sterility; after inoculation, they were incubated at 22 or 30° C. for 14 days when the *p*H values were compared with those of incubated controls. At the end of the experiment the growth and purity of the cultures were checked by transfer of loopfuls to ordinary agar slopes. It is probable that the values for the lower limiting *p*H are, in certain cases, slightly higher than they should be owing to the difficulty of detecting growth by small alterations in the *p*H value of a highly buffered medium. In a few cases, evidence of growth was obtained by the agar-slope method in tubes the *p*H of which did not differ significantly from that of the incubated controls; generally, however, there was fair agreement between the results of the two tests.

(11) *Formation of catalase.* Medium: Y.T.B., incubated for about 1 week at 22 or 30° C. The cultures were examined for evidences of growth (turbidity and change in *p*H) and adjusted to *p*H 7·0. Catalase was determined in a modified Lind's apparatus ((3), p. 125) in which the medium was held at 30° C. for 2 hr. with diluted hydrogen peroxide adjusted to *p*H 7·0.

(12) *Reduction of nitrate.* Media: Y.T.B.+0·1% nitrite-free potassium nitrate and Y.T.B.+0·002% potassium nitrite incubated at 22 or 30° C. for 1 week and tested by the methods described elsewhere ((3), p. 193). When no nitrite was found in the first medium, it was tested for nitrate and the second medium was tested for nitrite.

(13) *Decomposition of aesculin.* Medium: Y.T.B.+0·5% aesculin incubated at 22 or

30° C. for 3–14 days and tested by the addition of ferric citrate (0·2 ml. of a 0·5% solution per ml. of medium). In certain cases 3 Y.T.B. was substituted for Y.T.B.

(14) *Fermentation reactions.* Medium: Y.T.B. + 2% of the compound to be tested. Concentrated solutions of arabinose, xylose, dextrose, laevulose, saccharose, maltose, lactose, raffinose, mannitol and salicin were sterilized by filtration through Seitz E.K. filters and added aseptically to the sterile basal medium in concentrations sufficient to yield a medium of the composition already referred to. In the controls the solutions of test substances were replaced by equal volumes of Seitz filtrates of distilled water. Media and controls were incubated overnight at 37° C. to check sterility. 2% of starch and of inulin were sterilized by heat in the basal medium; in these cases uninoculated tubes of the media were used as controls. Duplicate tubes of the various media (including controls except where otherwise stated) were inoculated, incubated at 22 or 30° C. for 14 days, and titrated with 0·1 *N* sodium hydroxide. The titration figures for incubated controls were deducted to give the data recorded in the tables.

Orla Jensen (17) has claimed that the sterilization of small quantities of pentoses in media containing yeast extract has an activating influence upon the fermentation of sugars by lactic acid bacteria. The effect of sterilizing 0·05% of xylose in medium Y.T.B. upon the fermentation of dextrose and lactose by representative strains was examined. No appreciable influence was noted either upon the quantity of acid produced from substances fermentable without addition or on the ability of strains to attack compounds not ordinarily fermented by them.

(15) *Formation of carbon dioxide from dextrose.* Attempts were made to demonstrate formation of carbon dioxide by cultivation of the strains in dextrose agar stabs incubated in an atmosphere of the gas. Negative results only were obtained.

For quantitative work the medium employed was 2 Y.T.B. + 2% dextrose (a concentrated solution, sterilized by heat, added aseptically). The sterility of the medium was checked by incubation overnight at 37° C. The determinations were made in Demeter's (5) apparatus containing 10 ml. medium and 5–10 ml. of 0·1 *N* (approx.) baryta. The media were inoculated, the baryta was added, the tubes were connected immediately and the rubber stoppers were waxed. Duplicate cultures were incubated for 7 days at 22 or 30° C. when the baryta tubes were titrated with 0·1 *N* sulphuric acid. At first great difficulty was experienced in obtaining consistent results. This was apparently due, at least partially, to incomplete absorption of carbon dioxide owing to the formation of a scum of carbonate on the surface of the baryta. It was found that the reliability of the method was markedly increased by shaking the tubes daily. Shaking had comparatively little effect upon the amount of carbon dioxide recovered from homofermentative types but, in the case of the heterofermentative forms, it greatly increased the values obtained. The following are typical results:

	Carbon dioxide recovered (mg. per 10 ml. medium)	
	Culture undisturbed	Culture shaken
Homofermentative *Lactobacillus*	2·42	3·63
Heterofermentative *Lactobacillus*	7·46	15·29
Homofermentative *Streptococcus*	2·86	3·41
Heterofermentative *Streptococcus*	5·61	14·96

By employing the same batch of medium, shaking the tubes daily and taking all possible precautions to ensure uniformity in technique, it was ultimately possible to obtain results which were fairly uniform within the various groups and, in the great majority of cases, permitted a satisfactory differentiation of types. At the end of the incubation period, the purity of the cultures was checked by an examination of the growths obtained when loopfuls of media were transferred to slopes of nutrient agar.

(16) *Formation of volatile and non-volatile acids.* Medium: 500 ml. of 2 Y.T.B. (*p*H 7·0) + 4% dextrose + 3% calcium carbonate. For the more active strains the constituents were

sterilized together; for the less active types the dextrose and calcium carbonate were sterilized separately and added aseptically to the sterile basal medium. Sterility of media and purity of cultures were verified by the methods already described. Incubation was at 22 or 30° C. for about 6 weeks. After incubation the cultures were poured through No. 2 Whatman filter paper, and the residue was washed and the filtrate adjusted to 500 ml. with distilled water.

For the determination of volatile acids, a definite volume of the filtrate was acidified with sulphuric acid and any precipitate which settled was filtered off. The volatile acids were then distilled in steam until successive 100 ml. portions of the distillate gave reasonably constant titration values with 0·1 *N* sodium hydroxide. As has been shown elsewhere(21), it is essential to carry out a second distillation to obtain a reliable estimate of the total volatile acids and, if the conditions of this second distillation are carefully standardized, it is possible to calculate the amounts of the individual acids present. This depends upon the fact that the rate at which a lower member of the series of fatty acids distils depends upon its molecular weight and constitution. Each acid has a distillation constant but this varies somewhat with the concentration of the acid, the time required for distillation and the presence of salts. Consequently, the design of the apparatus and the technique must be rigidly adhered to in order to get comparable results.

In the present investigation, the procedures proposed by Dyer(6) and Wiegner(26) were followed as closely as possible. In the former, the distillation is carried out at a constant volume and the titration of the first and second 100 ml. portions of distillate provides sufficient data to calculate the total acids present and the actual amounts of acetic and butyric acids. In a recent paper, Clark & Hillig(2) have introduced certain refinements and greater precision in this method and obtained rather higher constants than those found by Dyer, but it was found(21) that Dyer's constants of 30·75 and 69·88, for acetic and butyric acid respectively, gave results agreeing closely with the figures obtained by complete distillation after making a correction for the final constant low value. In only three cases was the calculated distillation constant significantly greater than 30·75, indicating that butyric acid was present. In all other cases, acetic acid seemed to account for the total acid.

Wiegner's method consists in distilling and titrating three successive 100 ml. quantities from the solution made up each time to 200 ml. From these data, the amounts of acetic and butyric acid present may be calculated from the constants 36·59 and 72·77. Virtanen & Pulkki(22) have criticized this procedure on the ground that the half-distillation constant for butyric acid decreases with decrease in the concentration of the acid; they therefore follow up the first half-distillation with a complete steam distillation of the residue in order to obtain a direct measure of total acids. In the present work, however, the concentration of acid in the solutions which were distilled never exceeded 0·1%, so that variations in the constants were negligible and the results agreed well with those obtained by the Dyer method(21). The amount of butyric acid, as calculated from the above constants, exceeded 3% of the total volatile acids in four cases out of sixty analyses by the Wiegner method; all these cultures, however, contained less than 0·003% butyric acid. The distillation constants in the remaining cases did not depart significantly from 36·6 so that the total volatile acids were calculated as acetic acid.

For the determination of non-volatile acid a small quantity of the filtrate from the culture was diluted somewhat and then evaporated to about a third of the volume to get rid of volatile compounds. The solution was then neutralized with sulphuric acid and treated as described in detail elsewhere(21) with copper sulphate and calcium hydroxide at about 46° C. and filtered. The lactic acid in an aliquot portion of the filtrate was determined by oxidation with acid permanganate at boiling-point and distillation of the acetaldehyde into excess of bisulphite. The recovery of lactic acid by this method is about 90% and an appropriate factor was used in calculating the final result.

(17) *Form of lactic acid produced.* Medium: 1% Gurr's peptone + 0·3% marmite (adjusted to *p*H 7·0) + 3% calcium carbonate, sterilized in the autoclave. Sufficient concentrated heat-sterilized dextrose solution to give 4% in the media was added aseptically after sterilization. Before inoculation, the media were incubated for 3–4 days at 30° C. to check sterility. They were inoculated from young dextrose bouillon cultures and incubated at 22 or 30° C. for 10 days to 2 months. The purity of the cultures was checked during and after incubation by the transfer of loopfuls of medium to ordinary agar slopes. After incubation the cultures were filtered through No. 2 Whatman paper. 20 ml. concentrated sulphuric acid were added to 300 ml. filtrate; the calcium sulphate formed was filtered off. The filtrate was extracted with six to eight 200 ml. portions of ether in a separating funnel. The ether was evaporated off and the residue was dissolved in a small volume of water and boiled with zinc oxide and charcoal until neutral to litmus. From one to three crops of crystals were obtained by evaporation of the filtrate; each crop was purified by three recrystallizations.

Several methods are available for distinguishing between the active and inactive forms of lactic acid. The zinc salt of the former is about three times as soluble in water as that of the latter at room temperature, so that simple observation when preparing a solution is often a sufficient indication. Many investigators have made use of the fact that the zinc salt of active lactic acid crystallizes with two molecules of water (i.e. 12·9% H_2O), whereas the zinc salt of the inactive acid contains three molecules of water (i.e. 18·2% H_2O). Orla Jensen(15), Allen & Harrison(1), Karström & Virtanen(13) and Fred *et al.*(8) have all employed this method or gone further and determined the percentage of zinc present, in order to identify the form of lactic acid produced.

It would seem, however, to be much simpler to observe the optical activity which at once distinguishes the active from the inactive form and the *d*- from the *l*-acid. This procedure is also advantageous when, as frequently happens, only a small quantity of the salt is obtained. The zinc salt of the active lactic acid is soluble to the extent of about 5 parts in 100 parts of water at 15° C. and $[\alpha]_D$ is $-7{\cdot}6$ for the salt of the *d*-acid. In other words, even a few ml. of the saturated solution in a decimeter tube gives a rotation of $-0{\cdot}38°$ which is not difficult to read on a good polarimeter. This method was used throughout the present investigation.

Results

The detailed study of the selected strains of true lactic acid bacteria by the methods already described permitted their subdivision into nine more or less distinct groups. Five of these belong to the lactobacilli, two to the streptococci and one each to the micrococci and sarcinae. All nine groups possess the following characteristics in common: They are Gram-positive and are capable of producing lactic and acetic acids from dextrose, they fail to liquefy dextrose gelatin and have not been observed to produce spores.

Lactobacilli. In addition to the characteristics possessed in common with the other true lactic acid bacteria, the lactobacilli examined were found to be catalase-negative rods which did not produce slimy growths in saccharose media. The five types distinguished, however, possessed no other features in common.

The biochemical characteristics of the three groups of homofermentative lactobacilli are given in Table 1. A study of this table indicates that, as sources of nitrogen for organisms of group I, yeast preparations were superior to casein media and nutrient bouillon; autolysed yeast was outstanding in this

Table 1. *Biochemical characteristics of homofermentative lactobacilli*

Group	Strain	Fermentation reactions ml. 0·1 *N* acid formed per 10 ml. medium: Arabinose	Xylose	Dextrose	Laevulose	Saccharose	Maltose	Lactose	Raffinose	Starch	Inulin	Mannitol	Salicin	Fermentation products of dextrose: CO_2 (mg. per 10 ml. medium)	Lactic acid: Form 1	Form 2	Form 3	%	Volatile acid as acetic (%)	Lactic acid / Acetic acid	Sources of nitrogen millions per ml.: Autolysed yeast	Yeast extract	Difco yeast extract	Peptic casein digest	Difco tryptone	Nutrient bouillon
I	*a*	8·10	0·40	6·05	9·70	6·20	6·25	6·30	5·75	0·15	6·75	5·70	5·45	3·63	r.	r.	r.	3·33	0·090	37·0	865	715	64	13	88	79
	b	7·90	0·55	6·25	8·65	6·40	6·15	6·15	5·55	0·40	7·70	5·50	5·20	3·41	r.	r.	–	3·06	0·057	53·7	586	342	178	12	130	118
	c	7·95	0·50	5·15	8·05	5·50	4·85	5·45	5·55	0·00	0·25	4·65	4·65	2·53	–	–	–	–	–	–	1170	650	116	11	97	30
	d	9·45	0·45	6·15	9·05	6·50	6·25	5·15	6·15	0·60	0·30	4·85	5·30	3·85	r.	r.	r.	3·36	0·050	67·2	583	184	194	12	148	69
	e	7·65	0·35	6·15	9·45	6·35	6·65	5·75	5·90	0·15	0·15	4·70	5·25	4·84	r.	r.	–	3·28	0·079	41·6	555	121	199	5	66	83
	f	7·65	0·55	6·55	9·20	6·20	6·25	4·35	5·00	0·35	0·35	5·10	5·60	4·40	r.	r.	–	3·26	0·079	41·3	151	117	126	4	145	59
II	*a*	5·65	0·50	4·55	5·80	3·55	0·20	2·75	0·00	0·05	0·15	0·00	2·70	4·07	r.	r.	–	2·89	0·093	31·1	686	47	4	<1	5	107
	b	5·55	0·50	4·10	5·60	3·65	0·10	2·60	0·00	0·10	0·25	0·00	2·40	3·85	r.	r.	–	3·53	0·106	33·3	668	137	2	<1	12	108
	c	4·30	0·50	3·85	5·20	3·65	0·10	2·35	0·00	0·05	0·25	0·05	3·20	–	–	–	–	–	–	–	481	265	12	<1	12	65
	d	6·00	5·85	4·60	6·00	3·75	4·25	3·30	0·10	0·05	0·15	0·05	2·30	–	r.	r.	–	2·99	0·087	34·4	99	91	<1	<1	6	41
III	*a*	0·35	0·20	4·70	4·35	4·20	3·75	0·20	0·10	0·45	0·50	0·00	0·05	4·40	r.	–	–	3·06	0·074	41·4	583	219	115	8	46	120
	b	0·40	0·30	2·80	3·00	2·15	2·15	0·00	0·00	0·30	0·50	0·10	0·05	1·98	–	–	–	–	–	–	45	83	16	<1	2	162
	c	0·50	0·30	3·20	2·25	1·50	1·95	0·05	0·05	0·45	0·60	0·05	0·00	2·64	–	–	–	–	–	–	46	87	35	<1	6	69
	d	0·25	0·35	3·40	3·40	2·35	3·00	0·00	0·00	0·00	0·25	0·50	0·00	2·70	3·52	r.	r.	–	3·42	0·029	117·9	68	332	3	20	46	303
	e	0·35	0·35	2·75	2·45	0·00	1·85	1·20	0·00	0·35	0·50	0·10	0·15	3·52	r.	–	–	3·12	0·055	56·7	93	127	37	6	17	305
	f	0·35	0·30	3·10	3·10	0·00	2·90	0·00	0·05	0·15	0·35	0·05	0·00	4·95	–	–	–	–	–	–	399	315	40	7	64	214
	g	0·30	0·20	2·85	3·45	0·05	3·35	0·00	0·00	0·20	0·40	0·00	0·10	4·18	–	–	–	–	–	–	132	156	19	2	33	163
	h	0·25	0·20	3·45	2·20	0·00	3·25	0·00	0·05	0·20	0·40	0·00	0·00	3·96	r.	r.	–	1·96	0·073	26·9	191	116	33	1	43	94
	i	0·20	0·35	3·35	3·50	0·00	2·45	0·00	0·00	0·35	0·50	0·10	0·50	4·29	r.	r.	–	1·67	0·087	19·2	72	123	69	13	18	139
	k	0·30	0·25	2·70	2·35	0·00	2·45	0·00	0·00	0·30	0·40	0·00	0·05	2·31	–	–	–	–	–	–	54	137	64	3	23	213
	l	0·25	0·20	2·30	1·60	0·05	1·40	0·00	0·00	0·20	0·45	0·00	0·00	3·85	–	–	–	–	–	–	43	102	13	<1	<1	196
	m	0·40	0·40	2·85	2·50	0·10	2·75	0·00	0·00	0·30	0·55	0·10	2·50	2·31	–	–	–	1·56	0·068	22·9	75	170	25	<1	64	167

respect. The lactobacilli of group I fermented arabinose, dextrose, laevulose, saccharose, maltose, lactose, raffinose, mannitol and salicin. Arabinose and laevulose were fermented more rapidly than the remaining compounds and, as a rule, the laevulose media were acidified more readily than those containing arabinose. Little carbon dioxide was produced from dextrose; the quantity never exceeded 5 mg. per 10 ml. of medium. Lactic acid of the inactive type was formed in amounts which in all cases exceeded 3·0%. The ratio of lactic to acetic acid was never less than 37. The organisms were non-motile rods, $0{\cdot}5 \times 0{\cdot}5$ to $4{\cdot}0\,\mu$, which occurred singly, in pairs and in short chains. Colonies on dextrose agar possessed the characteristics usually associated with lactic acid bacteria. They were mainly in the medium and fusiform to irregularly spherical in shape with an entire edge; surface colonies were round, smooth and uniform. The diameter of the colonies in the medium reached 1·5–2·0 mm. Growths of organisms of this type were pale yellowish in colour; they were enhanced by the presence of dextrose in the medium. In milk an acid curd was formed; growth in milk was stimulated by the addition of dextrose and in more marked degree by that of Difco yeast extract. As a rule, methylene blue milk cultures showed slight reduction at the base of the tubes. When first isolated the majority of the strains produced a more or less pronounced reduction of nitrate but after they had been in pure culture for some time this property was lost. Aesculin media were blackened by all the cultures examined. The optimum temperature was about 30° C. and growth took place at a *p*H as low as 3·5.

The characteristics of the homofermentative lactobacilli have been reviewed recently by Pederson (18). The strains of group I are in almost perfect agreement with his description of *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.* The above description also agrees closely with the characteristics of *L. pentosus* and *L. arabinosus* (particularly the latter) as given by Fred *et al.* (7); according to Pederson these are synonyms of *L. plantarum*. It is also in good agreement with the data published by Orla-Jensen (16) for certain of his strains of *Streptobacterium plantarum*; preference for saccharose and maltose as compared with lactose is not, however, pronounced in the strains described in this paper.

The lactobacilli of group II differed from those of the first group in the following respects: In most media they grew less vigorously; growth in Difco yeast extract, peptic casein digest and Difco tryptone was poor. They did not curdle milk and the addition of dextrose produced only slight stimulation of growth. Methylene blue and nitrate were not reduced. Sugars were fermented less actively and, under the conditions prevailing in these experiments, raffinose and mannitol were not attacked. The ratio of lactic to acetic acid formed from dextrose was slightly lower. The optimum temperature was about 22° C. and growth could be initiated at a *p*H of 4·1 but not at 3·8. The majority of these differences are probably of comparatively little significance. It would certainly be inadvisable to attach importance to the differences in

fermentative capacity which have been recorded owing to the well-recognized variability of such characteristics. Meanwhile, therefore, this type should probably be classified with group I.

The third group of homofermentative lactobacilli exhibits pronounced differences from the other two types. Perhaps its most striking characteristic is its motility. A careful examination of hanging-drop preparations, made from young dextrose bouillon cultures, indicated that the great majority of the cells were non-motile but that a few exhibited evidences of motility. Generally movement was in the direction of the long axes of the cells, which showed little tendency to turn over. In certain cases the proportion of motile cells increased after the cultures had been passed through a few transfers in semi-solid agar, but macroscopic examination of cultures in this medium was valueless for detection of motility. In young dextrose agar slope cultures flagella were stained by Gray's method. Most of the cells possessed a single flagellum apparently originating either at the end or at one "corner". Some cells possessed two or three lophotrichic flagella. The flagella were generally of moderate length; in some cases, however, they did not exceed the length of the cells. The latter varied in shape from coccoid forms to rods. Chains consisting of elements varying from cocci to rods were common; chains as well as individual rods were frequently bent. The size of the cells varied from 0·3–0·5 by 0·3–4·0 μ; filaments were also observed.

The colonies were characteristic and distinct from those of the other groups. Surface colonies on dextrose agar were generally translucent and round to irregular in shape; colonies of the latter type showed a wavy to lobate edge. On microscopic examination the colonies appeared granular with a conglomerate centre. Deep colonies were fusiform to irregular. Microscopically they seemed to be exceptionally dense and generally showed small wart-like excrescences; some possessed peculiar greyish haloes. Deep colonies did not, as a rule, exceed 1·0 mm. in diameter.

In litmus milk little or no acid was formed. In certain cases growth in milk was slightly stimulated by the addition of Difco yeast extract but the addition of dextrose was generally without appreciable effect. Methylene blue and nitrate were not reduced but aesculin was blackened by the majority of the strains studied. As a rule, nutrient bouillon was the best of the sources of nitrogen examined but it was followed closely by yeast extract and autolysed yeast. The remaining sources of nitrogen tested were inferior. The only compounds fermented by all strains were dextrose, laevulose and maltose but the amounts of acid formed from these were comparatively small. The percentage of lactic acid produced from dextrose by the strains tested varied from 1·56 to 3·42 and the ratio of lactic to acetic acid from 19·2 to 117·9. The lactic acid was of the inactive type. The data suggest that it may be possible to divide strains of this type into high-and low-ratio subgroups. A subdivision on these lines would in most cases show some correlation with the fermentation reactions of the strains concerned. For the organisms of

group III the optimum temperature was about 30° C. Strains were capable of producing growth in artificial media the *p*H of which was 4·1.

A study of the above description indicates that the organisms belonging to group III are of an unusual type. There appears to be little doubt, however, that they should be classified with the lactobacilli; catalase-negative, Gram-positive, rod-shaped bacteria capable of producing from 1·5 to 3·5% of inactive lactic acid must be regarded as belonging to this genus. It is also comparatively easy to justify their inclusion among the homofermentative types. Some produce almost exclusively lactic acid and even for strains *h*, *i* and *m* the lactic-acetic acid ratios are higher than those recorded for the heterofermentative lactobacilli (groups IV and V). Confirmatory evidence is furnished by the data for formation of carbon dioxide; the values never exceed 5 mg. per 10 ml. medium. Among the homofermentative lactobacilli hitherto described, the characteristics of group III are unique. In heterofermentative types, motility has been described (8,25), but so far as is known this is the first record of motility in a high ratio form. The description of the colonies and the fact that nutrient bouillon is preferred to yeast preparations also shows that the organisms referred to do not belong to the common types of lactobacilli.

Table 2 contains the data on the biochemical characteristics of the heterofermentative lactobacilli. The latter were divided into two groups, IV and V. Both groups consisted of non-motile rods which occurred singly, in pairs or in short chains and failed to reduce nitrate or curdle milk. In litmus milk some acidity was produced but the rate of acidification was not appreciably increased by the addition of dextrose and only slightly by that of Difco yeast extract. Methylene blue was not reduced.

In group IV the rods were generally 0·5–0·7 × 0·7–6 μ, but filaments were also formed. The colonies on dextrose agar were of the type described for group I. Deep colonies in some cases reached 1·3 mm. in diameter. The presence of dextrose increased the rate of growth. The optimum temperature was about 30° C. and development took place in media of *p*H 3·5. Aesculin was not blackened. Autolysed yeast proved to be the best of the sources of nitrogen tested. On the average yeast extract, Difco yeast extract and tryptone and nutrient bouillon were of approximately equal value. Peptic casein digest was inferior. Arabinose, xylose, dextrose, laevulose, maltose and mannitol were fermented. The titration figures for xylose were the highest recorded for this group; they were followed in order by those for arabinose and laevulose. The high carbon dioxide values (13·75–22·66) and comparatively low lactic-acetic acid ratios (7·3–13·8) furnished the basis upon which these bacilli were included among the heterofermentative types. The lactic acid was predominantly the inactive form and was produced in amounts varying from 1·11 to 1·49%.

Pederson (19) has recently reviewed the literature on heterofermentative lactobacilli and has proposed a classification of these organisms. The characteristics of group IV are in excellent agreement with those of his *Lactobacillus*

Table 2. *Biochemical characteristics of heterofermentative lactobacilli*

Group	Strain	Fermentation reactions ml. 0·1 *N* acid formed per 10 ml. medium												Fermentation products of dextrose							Sources of nitrogen millions per ml.					
															Lactic acid											
		Arabinose	Xylose	Dextrose	Laevulose	Saccharose	Maltose	Lactose	Raffinose	Starch	Inulin	Mannitol	Salicin	CO_2 (mg. per 10 ml. medium)	Form 1	Form 2	Form 3	%	Volatile acid as acetic (%)	Lactic acid / Acetic acid	Autolysed yeast	Yeast extract	Difco yeast extract	Peptic casein digest	Difco tryptone	Nutrient bouillon
IV	*a*	9·30	11·95	5·25	8·00	0·05	6·10	0·15	0·00	0·15	0·10	2·40	0·25	14·85	r.	–	r.	1·48	0·132	11·2	750	265	13	2	155	153
	b	9·35	11·25	4·50	7·35	0·05	5·80	0·00	0·00	0·20	0·10	1·90	0·20	22·66	–	–	–	–	–	–	755	184	18	1	211	149
	c	9·65	12·00	4·30	7·50	0·05	5·55	0·00	0·05	0·10	0·00	2·70	0·20	13·75	r.	–	–	1·49	0·108	13·8	467	198	202	2	233	145
	d	9·60	12·05	4·85	7·20	0·00	5·50	0·00	0·00	0·05	0·00	3·05	0·30	14·19	–	–	–	–	–	–	632	41	105	4	84	184
	e	8·95	12·55	4·50	7·10	0·10	5·65	0·10	0·05	0·05	0·10	2·40	0·35	13·75	r.	–	–	1·11	0·153	7·3	395	91	146	3	226	118
	f	8·60	11·20	4·40	6·75	0·05	5·45	0·05	0·00	0·00	0·15	2·40	0·10	15·29	r.	r.	–	1·26	0·142	8·9	602	127	120	2	224	107
V	*a*	6·95	6·65	4·10	4·90	0·05	4·35	0·00	0·00	0·20	0·25	0·65	0·15	8·14	r.	r.	–	1·52	0·093	16·3	2	61	15	<1	2	68
	b	1·05	1·85	0·90	0·75	0·10	1·05	0·00	0·00	0·10	0·10	0·25	0·15	9·35	–	–	–	0·16	0·051	3·1	37	17	15	<1	10	28
	c	3·05	2·00	0·80	0·90	0·00	1·05	0·00	0·00	0·10	0·20	0·05	0·05	4·29	–	–	–	0·24	0·034	7·1	9	15	42	1	10	43
	d	1·20	1·65	0·45	0·95	0·10	0·50	0·10	0·10	0·10	0·20	0·20	0·10	7·81	–	–	–	–	–	–	9	24	6	<1	23	56
	e	0·70	1·00	0·75	0·65	0·00	0·80	0·05	0·00	0·20	0·25	0·15	0·10	7·59	–	–	–	–	–	–	4	30	<1	6	20	70
	f	1·15	1·75	3·25	0·75	0·05	0·80	0·15	0·10	0·15	0·25	0·25	0·10	8·69	–	–	–	–	–	–	9	48	7	2	30	102
	g	0·85	1·45	0·75	0·85	0·10	1·30	0·10	0·05	0·30	0·25	0·40	0·05	6·82	–	–	–	–	–	–	<1	21	<1	<1	5	26
	h	1·10	2·25	0·95	0·95	0·05	1·25	0·05	0·10	0·25	0·20	0·30	0·05	7·48	–	–	–	0·15	0·103	1·5	3	4	4	<1	9	48
	i	2·60	1·50	1·95	0·85	0·05	1·00	0·05	0·05	0·20	0·35	0·00	0·05	4·18	–	–	–	0·05	0·045	1·1	2	12	35	<1	7	39
	k	1·25	1·60	0·85	0·90	0·10	0·90	0·05	0·05	0·25	0·15	0·20	0·05	7·15	–	–	–	–	–	–	3	22	2	<1	30	67
	l	2·00	1·00	0·75	0·20	0·15	0·75	0·05	0·00	0·20	0·20	0·00	0·00	6·05	–	–	–	–	–	–	33	36	54	<1	12	97

brevis (Orla-Jensen) Bergey *et al.* which includes *L. pentoaceticus*. The description of the latter published by Fred *et al.*(7a) is also in good agreement with the data for this group.

Organisms *b–l* of group V were rods 0·3–0·5 × 1–10 μ or more; filaments up to 30 μ in length were common. Growth on most media was poor; deep colonies in dextrose agar did not exceed 0·5 mm. in diameter. Surface colonies on dextrose agar were irregular in shape and frequently possessed wisp-like edges; they were bluish and translucent. Deep colonies were fusiform to irregular and often showed wisps of hair-like outgrowths. The optimum temperature was nearer 22 than 30° C. and the bacilli were capable of initiating growth at a *p*H of 3·5. In the various sources of nitrogen tested development was poor and frequently erratic. It is, therefore, difficult to generalize regarding the relative merits of these media. Peptic casein digest was decidedly inferior to the others. Aesculin was blackened. Arabinose, xylose, maltose, dextrose and in almost all cases also laevulose were fermented but the amounts of acid formed from these sugars were small. From 4·18 to 9·35 mg. of carbon dioxide were produced by 10 ml. medium and these figures taken in conjunction with the data on lactic-acetic acid ratios (1·1–7·1) place the organisms among the heterofermentative types. The percentages of lactic acid were low and never exceeded 0·25; the form of the acid was not determined.

It is difficult to decide whether the organisms of group V should be classed as distinct types or merely as degenerate forms of group IV. The fact that strains were frequently obtained from the older samples of silage may be held to support the latter view. Groups IV and V possess a number of characteristics in common; these have been referred to among the general characteristics of the heterofermentative lactobacilli. The fermentation data for the two groups reproduced in Table 2 also bear a general resemblance; the sugars fermented are the same but the amounts of acid produced from these are smaller in the case of the group V than in the group IV strains. The characteristics of strain *a* (group V) may be mentioned in support of the view that the two groups are related. This strain bears a close resemblance to group IV in its fermentation characteristics; on the other hand its growth in yeast and casein media and in nutrient bouillon is of the meagre type which characterizes the other members of group V. Strain *a* has also been observed to form colonies resembling those of the other members of this group. It can, therefore, be regarded as an intermediate form between group IV and the remaining strains of group V. The existence of such intermediate strains may be held to support the view that groups IV and V are distinct, though possibly related, as well as that which regards V as degenerate forms of group IV. There is, however, some difficulty in reconciling the ability of group V (*b–l*) to decompose aesculin with the latter hypothesis; it seems improbable that in the process of degeneration an additional characteristic such as this should have been acquired. The differences in the form of the colonies are also difficult to bring into line with the degeneration hypothesis. It appears not improbable that

organisms of this type will ultimately prove to be similar to the inactive group briefly referred to by Pederson (19). Until, however, methods of cultivation capable of ensuring more abundant growth are devised and more is known about their characteristics, it seems inadvisable to attempt to identify them.

Streptococci. Two types of streptococci, one homofermentative, the other heterofermentative, were isolated from the samples of A.I.V. silage examined. These possessed the following characteristics in common: The cells were non-motile coccoids, ovoids and very short rods and occurred in pairs, short chains and in groups. Colonies were of the type common to so many of the lactic acid bacteria and already described for group I; the presence of dextrose in the medium stimulated growth. The optimum temperature was nearer 22 than 30° C. and growth could be produced in media of *p*H 4·1. Catalase was not formed, aesculin was blackened and nitrate was not reduced but methylene blue was more or less completely decolorized. Both types of streptococci differed from the other organisms examined in producing a comparatively vigorous development in peptic casein digest (Table 3).

Three of the streptococcus strains examined proved to be of the homofermentative type and constitute group VI. The cells of these organisms were generally $0{\cdot}3 \times 0{\cdot}3$–$0{\cdot}5\,\mu$. Deep colonies in dextrose agar did not exceed 0·7 mm. in diameter and the growth was yellowish white in colour. In milk an acid curd was formed; curdling was preceded by reduction of the litmus. Additions of dextrose and of Difco yeast extract increased the rate of coagulation of the casein. Methylene blue was reduced but no slime was formed in saccharose media. As a source of nitrogen peptic casein digest was inferior only to autolysed yeast; it was followed closely by nutrient bouillon. The compounds fermented included arabinose, xylose, dextrose, laevulose, maltose, lactose, mannitol and salicin. One of the strains was found to be capable of fermenting starch. About 3·25% of lactic acid was formed from dextrose; the acid was almost exclusively the *d*-form. The lactic-acetic acid ratio was about 80; in accordance with this observation the quantity of carbon dioxide formed was low, varying from 3·30 to 3·74. While the data quoted are sufficient to allow these organisms to be placed in the homofermentative subdivision of the streptococci, they are inadequate for purposes of identification. In further studies of group VI, therefore, the criteria recently recommended by Sherman and his collaborators (20) for the differentiation of the streptococci have been utilized. All of the strains examined were found to be incapable of producing β-haemolysis in blood-agar plates. They also failed to hydrolyse hippurate, to grow in the presence of 6·5% of sodium chloride or at 45° C. and to ferment glycerol and sorbitol. Ammonia was formed in 4% Bacto peptone, however, and the strains grew in the presence of 0·1% methylene blue. These characteristics and the rapid reducing action in litmus milk appear to be sufficient to justify the inclusion of the organisms in the *Streptococcus lactis* group.

The group of heterofermentative streptococci (VII) consists of four strains. In this case the cells were coccoids, ovoids and short rods, $0{\cdot}3$–$0{\cdot}5 \times 0{\cdot}3$–$2{\cdot}0$ or

Table 3. *Biochemical characteristics of streptococci*

Group	Strain	Fermentation reactions ml. 0·1 *N* acid formed per 10 ml. medium												Fermentation products of dextrose							Sources of nitrogen millions per ml.						
															Lactic acid											
		Arabinose	Xylose	Dextrose	Lævulose	Saccharose	Maltose	Lactose	Raffinose	Starch	Inulin	Mannitol	Salicin	CO_2 (mg. per 10 ml. medium)	Form 1	Form 2	Form 3	%	Volatile acid as acetic (%)	Lactic acid / Acetic acid	Autolysed yeast	Yeast extract	Difco yeast extract	Peptic casein digest	Difco tryptone	Nutrient bouillon
IA	*a*	4·60	4·80	4·80	5·15	3·75	4·40	3·75	0·20	1·70	0·10	2·95	3·45	3·41	d.	d.	d.	3·27	0·039	83·8	1161	322	193	437	314	584
	b	5·05	4·45	4·10	4·50	0·05	3·70	2·70	0·00	0·20	0·15	2·75	3·10	3·74	d.	d.	r.	3·28	0·042	78·1	718	465	203	482	67	451
	c	4·90	4·15	4·15	4·60	0·10	4·35	3·15	0·05	0·00	0·05	2·65	3·10	3·30	–	–	–	–	–	–	323	327	197	511	174	206
VII	*a*	7·20	2·90	5·85	7·85	5·85	5·65	3·50	3·75	0·35	0·15	0·35	3·90	15·51	l.	l.	–	1·58	0·070	22·6	768	269	185	75	122	86
	b	0·30	2·95	6·15	8·00	5·80	5·55	2·50	4·10	0·25	0·05	0·55	4·65	19·69	–	–	–	–	–	–	310	29	221	46	89	309
	c	7·85	2·10	4·90	7·45	5·85	4·70	4·15	3·90	0·40	0·15	0·35	0·10	14·96	l.	l.	r.	1·49	0·059	25·3	437	87	326	151	92	330
	d	4·25	5·55	3·00	5·85	3·05	3·20	2·40	3·20	0·05	0·20	0·10	1·05	20·90	–	–	–	–	–	–	744	157	54	111	169	170

even 3·0μ. Deep colonies in dextrose agar sometimes attained a diameter of 1·5 mm. The addition of saccharose to nutrient agar increased the size of the growths obtained, but the latter were not slimy. Abundant and very characteristic slimy growths were, however, produced in saccharose gelatin stabs. Milk was acidified but not curdled. The rate of acidification was slightly increased by the addition of dextrose and more markedly by that of Difco yeast extract which permitted the organisms to bring about coagulation of the casein. Methylene blue milk showed partial reduction at the base of the tubes. Autolysed yeast was the best of the sources of nitrogen; nutrient bouillon occupied second place. A fair growth was supported by peptic casein digest. Xylose, dextrose, laevulose, saccharose, maltose, lactose and raffinose were fermented by all strains and arabinose and salicin by most. Laevulose was fermented more actively than dextrose. The values for carbon dioxide varied from 14·96 to 20·90 and the lactic-acetic acid ratios were 22·6 and 25·3. Lactic acid was mainly the *l*-form and was produced in amounts of 1·49 and 1·58%.

Hucker & Pederson (12) have made a special study of the heterofermentative streptococci. They suggest the generic name *Leuconostoc* van Tieghem for organisms of this type and distinguish three species. The description given above is in agreement with their characterization of *Leuc. mesenteroides* (Cienkowski) van Tieghem.

Micrococci and sarcinae. The strains of micrococci isolated from A.I.V. silage form a very homogeneous group (VIII). Their biochemical characteristics are shown in Table 4. The cultures investigated formed from 3·29 to 3·45% of inactive lactic acid from dextrose. The ratio of lactic to acetic acid varied from 34·2 to 101·5 and the values for carbon dioxide from 1·43 to 2·86. The organisms must, therefore, be placed among the homofermentative types. Of the sources of nitrogen investigated, autolysed yeast was outstanding. Next in order came yeast extract, tryptone and nutrient bouillon, which were of approximately equal value. Difco yeast extract and peptic casein digest were inferior, particularly the latter. The fermentation reactions showed striking uniformity. Arabinose, xylose, dextrose, laevulose, maltose, lactose and salicin were fermented by all strains. The titration values for arabinose, xylose and laevulose were in all cases higher than those for the other compounds; the lowest values were those for lactose. Catalase was formed by all strains and aesculin was blackened, but neither nitrate nor methylene blue was reduced. No slime was formed in saccharose media. Some of the strains when first isolated were capable of coagulating the casein of milk but this property was rapidly lost. It was, however, restored by the addition of Difco yeast extract. The addition of dextrose to milk increased the rate of acidification only slightly. The optimum temperature was nearer 37 than 30° C. and the organisms were capable of growing in a medium the *p*H of which was 3·8. The cells were spherical and generally 0·5–1 μ in diameter. They were non-motile and occurred in irregular groups and other arrangements characteristic of micrococci. The

Table 4. *Biochemical characteristics of micrococci and sarcinae*

Group	Strain	Fermentation reactions ml. 0·1 *N* acid formed per 10 ml. medium: Arabinose	Xylose	Dextrose	Laevulose	Saccharose	Maltose	Lactose	Raffinose	Starch	Inulin	Mannitol	Salicin	Fermentation products of dextrose: CO_2 (mg. per 10 ml. medium)	Lactic acid: Form 1	Form 2	Form 3	%	Volatile acid as acetic (%)	Lactic acid / Acetic acid	Sources of nitrogen millions per ml.: Autolysed yeast	Yeast extract	Difco yeast extract	Peptic casein digest	Difco tryptone	Nutrient bouillon
VIII	*a*	6·35	6·40	4·40	6·85	0·00	4·95	3·20	0·00	0·05	0·00	0·05	4·15	2·42	r.	r.	–	3·29	0·047	70·0	1396	292	39	<1	176	178
	b	6·80	6·45	4·45	6·80	0·00	5·00	3·40	0·00	0·00	0·00	0·00	4·05	2·86	–	–	–	–	–	–	1338	403	68	1	203	96
	c	6·30	6·60	4·45	6·80	0·00	5·35	3·10	0·00	0·30	0·00	0·00	4·45	1·54	–	–	–	–	–	–	1458	242	34	<1	253	246
	d	6·65	6·90	4·65	6·85	0·00	5·65	3·20	0·10	0·25	0·05	0·05	4·55	2·09	–	–	–	–	–	–	1429	251	39	1	186	106
	e	6·55	7·00	4·70	6·90	0·25	5·70	3·15	0·20	0·00	0·10	0·20	4·65	2·31	r.	r.	–	3·35	0·033	101·5	1091	316	86	<1	224	165
	f	6·00	6·60	4·35	6·65	0·00	5·55	3·35	0·00	0·25	0·10	0·00	4·70	1·43	–	–	–	–	–	–	1485	208	49	3	174	324
	g	6·75	6·75	4·40	6·70	0·05	5·40	3·25	0·00	0·15	0·05	0·00	4·60	2·64	r.	r.	r.	3·45	0·101	34·2	1381	290	78	1	275	123
	h	6·35	6·75	4·40	6·65	0·00	5·45	3·40	0·05	0·20	0·05	0·00	4·50	1·87	–	–	–	–	–	–	1371	248	37	1	234	65
	i	6·90	6·40	4·45	6·60	0·00	5·55	2·70	0·00	0·10	0·00	0·05	4·80	1·87	–	–	–	–	–	–	1370	147	17	2	168	351
	k	6·55	6·95	4·55	7·10	0·15	4·85	3·65	0·10	0·15	0·00	0·10	4·50	2·42	–	–	–	–	–	–	1240	184	46	3	212	333
	l	6·40	6·65	4·75	6·75	0·05	5·70	3·65	0·10	0·10	0·00	0·00	4·40	2·75	r.	r.	r.	3·39	0·073	46·4	1285	232	47	3	197	356
	m	7·05	6·65	4·60	6·15	0·00	5·50	2·95	0·00	0·25	0·05	0·00	4·75	2·86	–	–	–	–	–	–	839	443	20	1	127	144
IX	*a*	0·05	0·05	2·40	2·20	0·00	2·80	0·00	0·10	0·00	0·15	0·00	0·85	0·66	–	–	–	–	–	–	5	64	23	<1	4	8
	b	0·25	0·30	2·85	2·50	0·00	2·00	0·10	0·10	0·25	0·15	0·05	2·65	0·66	–	–	–	–	–	–	1	32	3	<1	<1	<1
	c	0·20	0·20	3·10	2·65	0·05	3·10	0·05	0·00	0·10	0·25	0·00	2·95	0·22	r.	r.	–	1·16	0·005	232·0	9	9	32	<1	2	7
	d	0·05	0·05	2·25	2·75	0·00	1·45	0·00	0·00	0·15	0·05	0·00	1·55	0·33	–	–	–	–	–	–	<1	1	2	<1	<1	<1
	e	0·10	0·25	2·90	3·20	0·00	2·05	0·05	0·00	0·10	0·10	0·00	2·35	0·55	–	–	–	–	–	–	<1	1	3	<1	<1	2
	f	0·35	0·35	2·00	2·75	0·30	1·65	0·55	0·10	0·40	0·30	0·05	1·00	0·33	–	–	–	–	–	–	<1	1	<1	<1	<1	<1
	g	0·05	0·20	3·25	4·30	0·15	4·60	0·65	0·00	0·15	0·20	0·00	4·60	0·66	r.	r.	–	2·89	0·035	82·6	30	239	<1	<1	<1	86
	h	0·25	0·25	2·20	2·00	0·10	2·25	0·40	0·05	0·30	0·20	0·10	1·95	0·33	–	–	–	–	–	–	<1	41	<1	<1	<1	3
	i	0·10	0·05	2·45	2·45	0·00	0·05	0·00	0·00	0·00	0·05	0·00	2·45	0·44	–	–	–	1·48	0·032	46·3	2	10	3	<1	14	1
	k	0·20	0·15	2·00	2·20	0·05	0·15	0·05	0·00	0·20	0·25	0·00	2·75	0·44	–	–	–	–	–	–	<1	8	8	<1	1	12
		0·20	0·20	2·00	1·55	0·00	0·15	0·15	0·05	0·00	0·05	0·10	0·15	0·44	–	–	–	0·83	0·017	48·8	15	140	35	<1	4	<1

colonies on dextrose agar were of the type commonly associated with the lactic acid bacteria (see group I). Deep colonies in this medium sometimes attained a diameter of 1·7 mm. The organisms grew better on dextrose agar than on nutrient agar.

When first isolated the majority of the strains of sarcinae (group IX) formed regular packets but after they had been in pure culture for some time the tendency to do so became less pronounced. In the latter case the cells occurred in pairs, fours and irregular groups of great density. They were non-motile and varied from 0·3 to 1·5μ in diameter. Most of the colonies in dextrose agar were formed under the surface of the medium. They were fusiform to irregular in shape, and many possessed irregular outgrowths arranged in groups or clusters. Deep colonies were generally minute and did not, as a rule, exceed 0·5 mm. in diameter. Surface colonies, when they occurred, were round to slightly irregular. Their margins were often wavy and some colonies appeared bluish by transmitted light. By reflected light growth was white in colour. In nutrient agar it was increased by the addition of dextrose to the medium. The optimum temperature was nearer 22 than 30° C. and development took place in liquid media of *p*H 3·8. Litmus milk was slightly acidified; the rate of acidification was increased by the addition of Difco yeast extract but only slightly by that of dextrose. The organisms were generally capable of curdling yeast extract litmus milk. Neither methylene blue nor nitrate was reduced but aesculin was blackened. A notable difference between the sarcinae and the micrococci was that the former were not observed to produce catalase although the majority of the strains made good growth in the medium employed. No slime was produced from saccharose.

The quantitative biochemical data for group IX are shown in Table 4. Growth in the various sources of nitrogen tested was poor and generally erratic and did not permit conclusions to be drawn regarding the relative merits of the media. Dextrose and laevulose were fermented by all strains and salicin by all but one; a number of strains also fermented maltose. In the case of those strains in which it was investigated the lactic acid formed was mainly of the inactive type; the percentage varied from just under 1 to just under 3. Little acetic acid was produced. The ratio of lactic to acetic acid varied from 46·3 to 232·0 and the values for carbon dioxide were negligible (0·22–0·66). These organisms must, therefore, be regarded as homofermentative.

The general characteristics of groups VIII and IX have doubtless many points in common with published descriptions of certain micrococci and sarcinae. Such descriptions, however, do not generally include the results of quantitative work on the products of fermentation and, in fact, few data of this nature are to be found in the literature on these organisms. In his monograph on the lactic acid bacteria, Orla-Jensen (16) devotes considerable space to the tetracocci (micrococci and sarcinae) studied by him. Although he reproduces no quantitative data on the fermentation products of his organisms, in the second English edition of his *Dairy Bacteriology* he makes

the statements that the tetracocci always produce less acid than the streptococci and that much of the acid formed is acetic acid. These statements certainly cannot be applied to the micrococci and sarcinae and to the streptococci described in this paper. No quantitative results are given by Orla-Jensen for the carbon dioxide producing capacity of his strains. It is of interest to note, however, that a strain of *Tetracoccus liquefaciens* obtained from him produced more than 15 mg. carbon dioxide per 10 ml. medium when tested by the methods used in this investigation. This figure is many times the values obtained for the organisms of groups VIII and IX. It harmonizes with Orla-Jensen's statement that much of the acid formed by the tetracocci was acetic acid; both facts suggest that the organisms studied by him were of the heterofermentative type.

The formation of almost 3·5% of lactic acid by members of group VIII and of 1–3% by group IX are surprising results. It has been generally assumed that organisms of this type are incapable of producing large quantities of lactic acid. Orla-Jensen (16) states that his tetracocci were "on the whole but weak acid formers", and that in many cases the quantity of lactic acid produced was so small that its nature could not be determined. On the other hand, organisms capable of producing from 1 to 3·5% of lactic acid must be regarded as highly active fermenters.

In his classification of lactic acid bacteria, Orla-Jensen attaches great importance to such characteristics as formation of catalase, reduction of nitrate and production of surface growth in stab cultures. The tetracocci are stated to be positive in all three characteristics. The micrococci and sarcinae studied in this investigation produced neither surface growth nor reduction of nitrate and the latter organisms also failed to form catalase. It should be noted, however, that in the tabulations of the characteristics of his strains, Orla-Jensen includes some organisms which are incapable of producing surface growth and reduction of nitrate. Formation of catalase, on the other hand, is regarded by him as a reliable characteristic by which the tetracocci may be distinguished from other cocci.

A comparison of the data obtained in this investigation with the statements and figures of Orla-Jensen suggests that the organisms of groups VIII and IX are bacteria of uncommon types. They are homofermentative organisms and form large quantities of lactic acid, whereas the tetracocci appear to be heterofermentative and feeble acid producers. The inability of the sarcinae to form catalase is also an important characteristic distinguishing these organisms from those of Orla-Jensen. Caution should be exercised, however, in the interpretation of such observations. Orla-Jensen is careful to state that he has carried out no systematic search for organisms belonging to the genus *Tetracoccus*. He, therefore, makes no claim to have found "even approximately, representatives of all species belonging thereto". Further, in the absence of quantitative data on products of fermentation, it is impossible to state in what manner many of the common micrococci and sarcinae would

behave when cultivated for long periods in a suitable sugar medium containing calcium carbonate. In this connexion it appears worthy of note that Hucker (11), in his study of the acid-proteolytic (gelatin-liquefying) cocci, found that certain strains of micrococci were capable of producing large quantities of inactive lactic acid. In the case of group IX, the situation is further complicated by the fact that the organisms grew poorly on the media employed for their characterization. The difficulty of interpreting results obtained under such conditions has already been noted in the case of group V.

Other organisms. Reference to Table 2 in the first paper indicates that certain of the samples of A.I.V. silage yielded considerable numbers of yeast-like organisms. A limited number of strains was purified by plating and studied by the methods generally employed in the characterization of bacteria but no detailed investigation of these organisms was made.

In artificial media, the cells formed were generally ovoid in shape and about $6 \times 4 \mu$ in size. Rather less than half of the strains examined were capable of producing ascospores. The latter were easily demonstrated in old dextrose agar or dextrose gelatin cultures stained with carbol fuchsin and decolorized by means of nigrosine ((3), p. 59). As a rule, growth on dextrose agar was abundant, whitish and opaque. Gelatin was not liquefied and milk was either rendered alkaline or acidified and curdled. No attempt was made to identify the organisms referred to.

In the previous paper, attention was also directed to the fact that micro-organisms present in certain of the silage samples studied were capable of producing gas when cultivated in dextrose-agar shake cultures. Strains of these bacteria were isolated and examined. They proved to be coliform organisms which produced a delayed fermentation of lactose in bile-salt broth. All of the strains examined were motile; the majority failed to liquefy gelatin or to ferment saccharose and were methyl red and indol negative but capable of producing acetylmethyl carbinol and utilizing citrate. They, therefore, appear to possess characteristics which have more in common with those of *Bact. aerogenes* than with those of *Bact. coli.*

DISCUSSION

The investigations described in this and the preceding paper indicate that the microflora of silage prepared by the addition of mineral acid to green fodder consists mainly of lactobacilli. Both homo- and heterofermentative types are found. The former are represented by strains of *Lactobacillus plantarum,* the latter by those of *L. brevis.* Among the homofermentative forms two additional types occur. One of these is motile and, although its colonies show certain distinctions in form from those usually associated with the lactic acid bacteria, it produces large quantities of lactic acid and only negligible amounts of volatile acids and carbon dioxide. This is believed to be a type of lactobacillus not previously described. The other homofermentative form is difficult to differentiate with certainty from *L. plantarum.* In

addition to *L. brevis*, an unidentified heterofermentative lactobacillus has been isolated; this possesses characteristics in common with the inactive group referred to by Pederson. Two types of streptococci have also been obtained. One of these is homofermentative and belongs to the *Streptococcus lactis* group, the other is heterofermentative and has been identified with *Leuconostoc mesenteroides*. Strains of unidentified micrococci and sarcinae have also been isolated. They have been shown to possess characteristics not hitherto associated with organisms of these types.

The only paper which contains references to the species of organisms isolated from silage made by acidification of fodder crops with mineral acids is one by Virtanen (23) on A.I.V. silage. It contains the statement that the chief organism present is a long rod (*Lactobacillus*) which forms lactic acid from both hexoses and pentoses. At an earlier stage the presence of acetic acid in the silage is ascribed to the activity of *Lactobacillus pentoaceticus* (= *L. brevis*). No mention is made of the occurrence of homofermentative lactobacilli. Virtanen notes that betacocci, yeasts and spores of *subtilis*, *mesentericus* and *amylobacter* are also found. He does not, however, give detailed descriptions of the characteristics of the microflora.

In the first paper of this series the stages at which the main groups of micro-organisms were found were briefly referred to. That statement can now be amplified by references to individual types. Strains of *L. plantarum* were obtained from old material as well as from that recently ensiled but most frequently from the former. On the other hand, all the strains of group II were isolated from a single sample 4 weeks old. The motile lactobacilli (group III) were most numerous in samples less than 6 weeks old but a few also occurred in the older material. *L. brevis* was never isolated from samples less than 8 weeks old, while the lactobacilli of group V were obtained from silage 4–10 weeks old. The fact that the streptococci occurred only in silage recently ensiled has already been noted (4). Micrococci (group VIII) have not been recovered from samples more than 4 weeks old; on the other hand, the sarcinae have been found mainly in the older samples. It appears, therefore, that the streptococci, micrococci and motile lactobacilli were found mainly in material recently ensiled while the majority of the lactobacilli and the sarcinae were associated with the older samples. The sequence streptococci-lactobacilli is one which is frequently observed in fermenting materials. Its occurrence is often attributed to the greater tolerance of the latter types of organisms to rising concentrations of hydrogen ions. According to Virtanen (24), however, the hydrogen-ion concentration in A.I.V. silage does not change appreciably. The comparatively rapid disappearance of the streptococci from the medium must, therefore, be attributed to other factors. Whether the rise in the concentration of lactate ions can be held to be responsible is also doubtful; Virtanen's data suggest that the rate of formation of lactic acid is too slow to account for these occurrences.

The outstanding features of the data on the sources of nitrogen employed

for the cultivation of the organisms studied are the excellent results obtained with autolysed yeast and the marked inferiority of peptic casein digest. It should be stated that the comparison of these media is not based strictly upon percentage of nitrogen as suggested by Orla-Jensen (16). Nitrogen determinations were carried out on media made at an earlier date by the same methods and the concentrations employed in the present investigation were selected to give approximately equal percentages of nitrogen on the assumption that media made by the same methods at different dates would not show appreciable variations in composition. It is difficult to believe, however, that variations due to this cause can account for the whole of the observed differences between autolysed yeast and casein digest. It is also unlikely that the explanation of the results recorded is to be found in the characteristics of the casein employed. Media prepared by digesting casein for 10 and for 63 days were compared but 63 days' digestion did not effect a marked or consistent increase in the counts obtained. In this connexion it should also be noted that, although the majority of the strains tested grew poorly in casein digest, the streptococci made good growth in this medium. It seems improbable, therefore, that the poor results yielded by it were due to the characteristics of the casein used. In his investigations, Orla-Jensen obtained better results with peptic casein digest than have been secured in these studies. The majority of his organisms were, however, obtained from dairy products.

In individual groups of organisms the fermentation reactions have been much more uniform and consistent than might have been anticipated. They have also shown remarkably close agreement with published data of other workers in the case of those types which it has been possible to identify. In particular, the relative rates of fermentation of the compounds tested have proved to be valuable characteristics for identification purposes. The superiority of one or both of the pentoses and of laevulose over the other substances manifests itself repeatedly and may almost be said to be characteristic of all those types of organisms which have made good growth in the media employed. On the other hand, the variation of group I in ability to reduce nitrate suggests that this is not a characteristic upon which the major subdivisions of the lactic acid bacteria should be based. The value of the catalase test for differentiating sarcinae from catalase-negative lactic acid bacteria also appears to be open to question.

For the differentiation of the organisms, the characteristics which have proved to be most useful are ability to produce carbon dioxide, percentage of lactic acid and the lactic-acetic acid ratio. The values obtained for the various groups are given in the table on p. 264.

The nearest approach to clear-cut results as between homofermentative and heterofermentative organisms is given by the lactic-acetic acid ratio. The only groups which show overlapping are III (*ii*) and VII. The homo- and heterofermentative types also show a more or less sharp differentiation in

their ability to produce carbon dioxide from dextrose. Some overlapping does, however, exist between group V and certain of the homofermentative types. The percentages of lactic acid formed from dextrose are also remarkably well separated. Under the conditions prevailing in these experiments, the majority of the homofermentative forms produced about 3% lactic acid whereas the heterofermentative organisms generally formed about 1·5% or less. The most notable exception to this rule was found in group IX—homofermentative sarcinae, many of which grew poorly in artificial media. It will be observed, however, that no one characteristic yields results which are of absolute differential value. In order to arrive at a differentiation of certain of the groups of organisms it is necessary to employ two or more characteristics.

	CO_2 mg. per 10 ml.	Lactic acid %	Lactic acid / Acetic acid
Homofermentative:			
Group I	2·53–4·84	3·06–3·36	37·0– 67·2
II	3·85–4·07	2·89–3·53	31·1 –34·4
III (*i*)	1·98–4·40	3·06–3·42	41·4–117·9
III (*ii*)	2·31–4·95	1·56–1·96	19·2– 26·9
VI	3·30–3·74	3·27–3·28	78·1– 83·8
VIII	1·43–2·86	3·29–3·45	34·2–101·5
IX	0·22–0·66	0·83–2·89	46·3–232·0
Heterofermentative:			
Group IV	13·75–22·66	1·11–1·49	7·3–13·8
V *a*	8·14	1·52	16·3
V *b–l*	4·18– 9·35	0·05–0·24	1·1– 7·1
VII	14·96–20·90	1·49–1·58	22·6–25·3

Summary

1. The microflora of A.I.V. silage consists mainly of lactic acid bacteria—lactobacilli, streptococci, micrococci and sarcinae. Some samples also contain yeasts and yeast-like organisms.

2. Among the lactobacilli, both homo- and heterofermentative types are represented. The former include strains of *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.*; in the heterofermentative group *L. brevis* (Orla-Jensen) Bergey *et al.* has been found.

3. Cultures of a motile homofermentative lactobacillus were isolated; this appears to be a new type. Among the unidentified heterofermentative lactobacilli, forms which may prove to be similar to the inactive group of Pederson were encountered.

4. The homofermentative streptococci were found to belong to the *Streptococcus lactis* group; the heterofermentative forms were identified with *Leuconostoc mesenteroides* (Cienkowski) van Tieghem.

5. Strains of unidentified homofermentative micrococci and sarcinae were isolated and shown to possess characteristics not usually associated with organisms of these types.

6. Streptococci, micrococci and motile lactobacilli were found mainly in fodder recently ensiled; the majority of the lactobacilli and sarcinae were associated with the older samples.

7. The characteristics which proved to be most valuable for the differentiations of the organisms were ability to produce carbon dioxide, percentage of lactic acid formed and lactic-acetic acid ratio.

REFERENCES

(1) ALLEN, L. A. & HARRISON, J. (1936). *Ann. appl. Biol.* **23**, 546–57.
(2) CLARK, E. P. & HILLIG, F. (1938). *J. Ass. off. agric. Chem., Wash.*, **21**, 684–8.
(3) CUNNINGHAM, A. (1934). *Practical Bacteriology*. Edinburgh: Oliver and Boyd.
(4) CUNNINGHAM, A. & SMITH, A. M. (1939). *Zbl. Bakt.* II, **100**, 394–408.
(5) DEMETER, K. J. (1930). *Zbl. Bakt.* **82**, 71–99.
(6) DYER, D. C. (1917). *J. biol. Chem.* **28**, 445–73.
(7) FRED, E. B., PETERSON, W. H. & ANDERSON, J. A. (*a*) (1921) *J. biol. Chem.* **48**, 385–412; (*b*) (1922) *J. biol. Chem.* **53**, 111–23.
(8) FRED, E. B., PETERSON, W. H. & DAVENPORT, A. (1919). *J. biol. Chem.* **39**, 347–84.
(9) —— —— —— (1920). *J. biol. Chem.* **42**, 175–89.
(10) GRAY, P. H. H. (1926). *J. Bact.* **12**, 273–4.
(11) HUCKER, G. J. (1928). *Tech. Bull. N.Y. St. agric. Exp. Sta.* no. 144.
(12) HUCKER, G. J. & PEDERSON, C. S. (1931). *Zbl. Bakt.* II, **85**, 65–114.
(13) KARSTRÖM, H. & VIRTANEN, A. I. (1937). *Ann. Acad. Sci. fenn.*, Ser. A, **48**, 28.
(14) KOPELOFF, N. & COHEN, P. (1928). *Stain Tech.* **3**, 64–70.
(15) ORLA-JENSEN, S. (1904). *Zbl. Bakt.* II, **13**, 435.
(16) —— (1919). *The Lactic Acid Bacteria*. København.
(17) ORLA-JENSEN, A. D. (1933). *J. Soc. chem. Ind.* **52**, 374–9 T.
(18) PEDERSON, C. S. (1936). *J. Bact.* **31**, 217–24.
(19) —— (1938). *J. Bact.* **35**, 95–108.
(20) SHERMAN, J. M. (1937). *Bact. Rev.* **1**, 1–97.
(21) SMITH, A. M. (1938). *Analyst*, **63**, 777–81.
(22) VIRTANEN, A. I. & PULKKI, L. (1928). *J. Amer. chem. Soc.* **50**, 3138–51.
(23) VIRTANEN, A. I. (1937). *Zbl. Bakt.* II, **95**, 472–7.
(24) —— (1938). *Cattle Fodder and Human Nutrition*, pp. 78–102. Camb. Univ. Press.
(25) WEINSTEIN, L. & RETTGER, L. F. (1932). *J. Bact.* **24**, 1–28.
(26) WIEGNER, G. (1926). *Anleit. z. quantitat. agrikulturchem. Prakt.* Berlin.

(*MS. received for publication* 29 *April* 1940)

547.979.8:636.2.085.3

263. THE PASSAGE OF CAROTENOIDS FROM FOOD TO MILK IN THE COW. THE FATE OF LYCOPENE

By A. E. GILLAM
From the Department of Chemistry, University of Manchester

and S. K. KON
From the National Institute for Research in Dairying, University of Reading

Introduction

It has long been known that the natural yellow colour of butter is mainly due to the plant-pigment carotene together with a small amount of a xanthophyll, probably lutein, and that these pigments are obtained solely from the food of the cow (Palmer & Eckles(1)). The fact that grass and other green leaves contain about twice as much lutein as carotene, whereas the milk fat pigment is mostly carotene, implies a very definite preferential absorption of the hydrocarbon carotene in the cow's digestive tract.

The importance of the natural colouring matter of butter only came to light with the discovery that the carotene, so characteristic of plants, could replace completely in the diet of animals the otherwise indispensable vitamin A of liver oils. We now know that the gross biological vitamin A activity of butterfat is due to the separate contributions made by the vitamin A precursor carotene, and the true vitamin A itself.

The fact that carotene occurs naturally in several isomeric forms of which the α-, β-, and γ-isomers are well authenticated (Kuhn & Lederer(2); Kuhn & Brockmann(3)) raised the question as to which isomer is present in butterfat. This query is the more pertinent because, of the three isomers, the β form is biologically twice as active as either of the other two. Although early chromatographic experiments indicated that the carotene of butter is the β form often mixed with appreciable quantities of the α-isomer (Gillam & Heilbron(4)), later experiments, in which the crystalline pigment was isolated, have shown that butter carotene is normally composed of the β-isomer with only minute amounts of α-carotene (Strain(5); Gillam & El Ridi(6)). It has since been found that when α-carotene is present in the diet, e.g. when carrots are fed, it passes readily into the milk and butter (Strain(7); Meigs(8)).

In view of the approximate parallelism that exists between the natural yellow colour of butter and its total vitamin A activity—especially in butter from cows of one breed—it is important to be assured that the yellow colour shall not be due on occasion to substances which, whilst imparting yellow colour to the butter, contribute nothing to the biological activity. Thus Quackenbush *et al.*(9) have shown that epiphasic yellow pigments, formed in lucerne silage by the action of acid and devoid of vitamin A activity, can be

recovered from the butter of cows receiving the silage. Another highly coloured but biologically inactive carotenoid is the carotene isomer lycopene—the pigment of the tomato. In an earlier examination of the butter pigments by the chromatographic method this carotenoid was detected by its very characteristic absorption spectrum in a casual sample of Danish butter but there was no evidence to show how the pigment came to be present (Gillam & Heilbron (4)). Examination of the carotenoid fractions of various butterfats by other workers has not disclosed the presence of lycopene either in cow's milk or in human milk even when tomatoes were included in the diets (Van Wijngaarden (10); Willstaedt & With (11); With & Friderichsen (12)).

In the present investigation it was decided to submit the question of the occurrence of lycopene in milk and butter to a further direct test by finding whether or not cows can pass lycopene into their milk (and hence to the butter) when their diet contains a good supply of this pigment.

EXPERIMENTAL

In the several experiments detailed below the general procedure has been to place a cow on a known diet and collect a series of milk samples, separate the fat by churning and determine the concentration of carotenoids and vitamin A by a spectrophotometric method (Gillam (13)). The cow was then fed with a lycopene-containing supplement of tomato purée, samples of milk again being collected, churned, and the resulting butter examined. In addition to an evaluation of the pre- and post-experimental butters for carotenoids and vitamin A the two samples were in each case subjected to chromatographic analysis. For this purpose they were saponified and their unsaponifiable matters extracted completely with peroxide-free ether. After transferring the solutes to petroleum ether the two samples were chromatographically adsorbed on separate columns of activated alumina in order to detect lycopene if present in the carotene. By absorbing the pre- and post-experimental samples side by side a stricter comparison was obtained.

Two preliminary experiments were carried out with stall-fed cows in February and early April respectively, when butter is low in carotenoids and when it might be expected that any influx of lycopene into the fat would be detected more easily.

Experiment 1

One cow, a pure-bred Shorthorn receiving a daily diet of 5 lb. hay, 10 lb. grass silage (containing 25 % dry matter), 30 lb. mangels, 2 lb. sugar-beet pulp and 14 lb. concentrate mixture, had tomato purée (1500 g.) added to its food. The purée was fed in four feeds on 7 February 1939 at 6.0 a.m., 10.0 a.m., 1.0 p.m. and 3.30 p.m., and in each case it was diluted with an equal volume of water, and poured over ½ lb. of the sugar-beet pulp. The mixture was allowed to soak for ½ hr. and in this form was readily taken by the cow. The total carotenoids in the purée equalled 22 mg. % as lycopene and chromatographic adsorption showed that 90 % of this was in fact lycopene together with traces

of carotene and xanthophylls, this being a typical composition for tomato purée (Kuhn & Grundmann (14)). The special grass silage consumed by this cow was quite high in carotene (25 mg. % on the dry basis): hence the total carotene content of the fodder was abnormally high for the time of the year. This was reflected in the relatively high vitamin A and carotene content of the butter. The tomato purée contained some 300 mg. of lycopene and thus contributed about as much total epiphasic carotenoids as were present in a day's supply of silage. The carotenoids present in the other foods were relatively insignificant. Aliquots were obtained of the daily milk yields of the cow on the two days preceding and the four days following the administration of tomato purée. Butter was churned from the unripened unpasteurized cream and, after rendering, the unsaponifiable matter of the fat was examined spectrophotometrically. The results are given in Table 1. They show no significant change in carotenoid or vitamin A content.

Table 1. *Spectrophotometric analysis of butterfats*

Type of fat	Daily fat yield g.	Total carotenoids 2200 basis, $CHCl_3$ mg. %	Vitamin A 1300 basis $CHCl_3$ mg. %
		Pre-experimental	
2nd day	232	0·322	1·14
1st day	254	0·311	1·34
Mean	243	0·316	1·24
		Post-experimental	
1st day	229	0·293	0·98
2nd day	237	0·314	1·17
3rd day	289	0·350	1·29
4th day	267	0·330	1·35
Mean	255	0·322	1·20

Chromatography of the pigments. Only 250 and 350 g., respectively, of the pre- and post-experimental samples were available, and the total pigment was only of the order of 1 mg. in each sample. Comparative adsorption of the unsaponifiable matter of both samples on activated alumina from petroleum ether-benzene gave no evidence of any difference between them, although lycopene was not definitely proved to be absent from either sample.

Experiment 2

One cow, a pure-bred Shorthorn whose feed consisted of 12 lb. of hay, 40 lb. mangels and 10 lb. concentrates with no other addition, was given during 3 April 1939 a supplement of 4100 g. of tomato purée divided into four doses. On the day of feeding the concentrates were cut down to 5 lb. As before, the tomato purée was fed mixed with some sugar-beet pulp. The total carotenoids fed were of the order of 750 mg. of which about 675 mg. was lycopene. As in the previous experiment butter samples were obtained on 2 days preceding and 4 days following the feeding. The pre- and post-experimental samples were blended for analysis. The results are given in Table 2.

Table 2. *Spectrophotometric analysis of butterfats*

Type of fat	Mean daily fat yield g.	Total carotenoids 2200 basis, $CHCl_3$ mg. %	Vitamin A 1300 basis, $CHCl_3$ mg. %
Pre-experimental	253	0·080	0·78
Post-experimental	265	0·127	1·10

It would appear that the carotene in the tomato purée increased the vitamin A and carotene content of the butter, which was very low in these factors and therefore susceptible of improvement. The post-experimental butterfat available (137 g.) represented only 0·17 mg. of hydrocarbon carotenoids which was insufficient to show with certainty whether lycopene was absent or not. Comparative adsorptions on alumina were, however, carried out with both the pre- and the post-experimental butter pigments and the chromatograms appeared to be indistinguishable.

After the experience gained in the two preliminary experiments it was decided to feed much larger quantities of tomato purée.

Experiment 3

This experiment was carried out in December. One cow, a pure-bred Shorthorn receiving a diet of 20 lb. mangels, 12–14 lb. hay and 12 lb. concentrates was fed with a supplement of tomato purée. 22½ lb. were given, mixed with some sugar-beet pulp, throughout the course of four days, in daily quantities of 4, 8, 8 and 2½ lb. respectively. This was readily consumed but on the last day of feeding it was noticed that the cow was unsettled and her milk yield decreased appreciably. The supplement contained 20·4 mg. % of total carotenoids. The gross unsaponifiable matter exhibited absorption bands in petroleum ether, b.p. 70–80°, at 504, 471 and 443·5 mμ, hence the pigment was mainly lycopene. Aliquot samples of fat were obtained from the pooled milk yield of the 3 days preceding the feeding of the purée and of 5 days after the feeding was started. Analytical results are given in Table 3. In this experiment there was no significant change in carotene content but a slight rise in vitamin A content.

Table 3. *Spectrophotometric analysis of butterfats*

Type of fat	Total carotenoids 2200 basis, $CHCl_3$ mg. %	Vitamin A 1300 basis, $CHCl_3$ mg. %
Pre-experimental	0·30	1·00
Post-experimental	0·33	1·35

Chromatography of the pigments. (*a*) *Pre-experimental butter.* 350 g. of butterfat were saponified with alcoholic potash and the unsaponifiable matter extracted exhaustively with ether, the extracts being carefully washed with several lots of water, dried, and the solutes transferred to petroleum ether. The whole extract was then adsorbed on a column of alumina (Merck's

activated, diluted with inactive material 1:2) of dimensions 20 by 2·8 cm. The chromatogram obtained is shown in Table 4, absorption maxima being for extracts in petroleum ether (b.p. 70–80°).

Table 4. *Chromatographic analysis of pre-experimental butterfat*

Length of zone cm.	Colour of zone	Absorption bands	Pigments present
1·0	Yellowish	None	Xanthophylls and oxidation products?
3·0	Scarcely coloured	—	—
1·0	Pale yellow	None	—
A 0·6	Brown	None	—
B 3·6	Bright yellow	None	—
3·6	Red-brown	480 and 451 mμ	β-Carotene
Washings	Yellow	477 and 448 mμ	Pseudo-α-carotene

The zones A and B were cut out separately, eluted with petroleum ether-alcohol and after removal of the alcohol were re-adsorbed from petroleum ether again. Zone A gave a very sharply defined brown band and a very pale yellow region above it. The latter on elution gave an extract exhibiting no selective absorption in the visible region but gave a strong blue colour with antimony trichloride in chloroform exhibiting well-defined absorption at 620 mμ typical of vitamin A. The sharply defined brown band yielded an eluate exhibiting no characteristic light absorption and was undoubtedly due to the artefact normally developed in vitamin A concentrates and made obvious when these are adsorbed on alumina or lime (Castle *et al.*(15)).

Fraction B was eluted and re-adsorbed. It yielded only one yellow zone which, on elution, again showed no selective light absorption but gave a strong violet-brown colour with antimony trichloride exhibiting absorption maxima at 605 and 575 mμ (artefacts?).

(*b*) *Post-experimental butter.* 400 g. of dry butterfat obtained from the bulked milk collected over the 5 days after feeding the tomato purée, were saponified and the unsaponifiable matter extracted as before and transferred to petroleum ether. To remove xanthophylls and vitamin A this was extracted seven times with equal volumes of 92 % methyl alcohol. The petroleum ether phase was washed with water, dried, and adsorbed on alumina as before. The main bulk of the pigment was contained in a red-brown zone (β-carotene, absorption maxima 451 and 481 mμ), whilst the yellow lower zone contained pseudo-α-carotene[1] (maxima 477 and 447 mμ). Above the β-carotene zone was a small amount of pigment in the form of a faint red-brown zone and a lower bright yellow zone. These were cut out together and eluted with petroleum ether containing a little alcohol and, after removal of the latter by washing with water, the dried petroleum ether extract was re-adsorbed on alumina.

[1] This pigment is an isomer of β-carotene and is produced spontaneously when solutions of the latter are heated. For properties of pseudo-α-carotene and the conditions required for isomerization cf. Gillam & El Ridi(16), Carter & Gillam(17) and Zechmeister & Tuzson(18).

It yielded two separate zones which on elution yielded distinctly yellow solutions,

Upper zone: maxima 471 and 441 mμ
Lower zone: maxima 451 and 425 mμ
} (solvent, petrol b.p. 70–80°),

possibly due to oxidation products of β-carotene (*vide infra*).

For comparative purposes a solution containing both β-carotene and lycopene in the approximate proportions of 5:1 was adsorbed on alumina. One adsorption was sufficient to give an obvious separation into a red-violet zone containing most of the lycopene and a larger red-buff zone containing the carotene. The absorption maxima after the first separation were 501, 470 and 438 mμ for the lycopene and 480 and 450 mμ for the carotene in petroleum ether b.p. 70–80°. A second adsorption was necessary to obtain each pigment completely free from the other.

There is thus no evidence of any lycopene having passed into the milk even when some 2000 mg. of pigment had been taken by the cow.

Examination of faeces. Since the food of the cow in this experiment definitely contained lycopene while the milk did not, it was decided to seek for this pigment in the faeces. A sample was accordingly collected in the course of the third day of tomato purée feeding. A 20 g. lot of the air-dried material was extracted in a Soxhlet apparatus, first with methyl alcohol and then with petroleum ether. The combined greenish yellow extracts were saponified overnight with cold alcoholic potash, the carotenoids driven into petroleum ether by addition of water and extracted with 90 % methyl alcohol, when some two-thirds of the total colour were removed by the aqueous alcohol. The unpurified petroleum ether phase exhibited clear absorption maxima at 499, 470 and 445 mμ, indicating that considerable lycopene was present, and after adsorption on alumina a broad red lycopene zone constituting the main bulk of the epiphasic carotenoids was obtained in the column (maxima, 544, 504 and 472 mμ in CS_2; 521, 485 and 455 mμ in benzene). It is thus abundantly clear that lycopene passes readily from the food into the faeces but not into the milk.

Examination of colostrum fats

In the course of our search for lycopene in butterfat we examined several colostrum fats, this particular variety of milk fat being chosen only because it contains more pigment per unit weight of fat than does ordinary butter. Brief details on two typical samples are of interest.

Colostrum fat 1. Old sample from a Guernsey cow. Collected in July 1936. The cow had been grazing until the day before calving. Total carotenoids, after some loss in storage = 3·3 mg. in 180 g. fat. The fat was saponified and the unsaponifiable matter extracted and transferred to petroleum ether. The first adsorption on alumina yielded pale-red and yellow-brown zones close together but above the β-carotene zone (maxima 453 and 423 mμ in petroleum ether). Re-adsorption gave a reddish zone above a yellow-brown one but the

two were not separable. There was about 0·17 mg. total pigment having absorption bands at 453 and 427 mμ. The pigment was eluted and re-adsorbed when two separate bands were obtained.

Upper band: rosy red zone which on elution gave a solution estimated to contain 0·06 mg. of pigment having maxima at 452 and 424 mμ.

Lower band: yellow-brown zone containing approximately 0·05 mg. of pigment having absorption maxima at 451 and 424 mμ.

Hence what might at first be taken to be a lycopene zone by the colour and location of the adsorbed pigment consistently gave absorption bands at much shorter wave-lengths, i.e. at 452 and 425 mμ in petroleum ether in contrast to values of 507, 474 and 466 mμ for pure lycopene in the same solvent.

Colostrum fat 2. Fresh sample collected 21 May 1939. The cow, a Shorthorn, had been grazing until the day before calving. Total carotenoids 2·6 mg. %; 210 g. of fat available = 5·5 mg. pigment. Unsaponifiable matter adsorbed on alumina. The first adsorption yielded a narrow red zone and another brown one close together but above the main carotene and pseudo-α-carotene zones. After cutting out and eluting the top two zones followed by re-adsorption on alumina from petroleum ether, the pigment yielded two quite separate rings on the column:

Upper ring: well-defined brownish-mauve, absorption maxima 451 and 425 mμ (petroleum ether).

Lower ring: bright yellow, maximum 450 mμ (425 mμ band?).

The nature of these pigments present in traces and having absorption maxima at such unusually short wave-lengths for carotenoids is at present unknown, although one of them might possibly be due to one of the many known oxidation products of carotene such as β-carotene oxide which happens to have a similar absorption spectrum (Euler *et al.*(19)). There was thus no evidence of the presence of lycopene in colostrum fats.

Summary

1. The question whether the tomato pigment, lycopene, an isomer of carotene, can pass from food into milk in the cow has been submitted to direct test by feeding three different cows with tomato purée and examining the resulting milk fats separately for lycopene. No evidence of the presence of lycopene has been detected in the unsaponifiable matter of the butter fat when this was subjected to careful chromatographic adsorption and it is concluded that, although the cow normally absorbs α- and β-carotene and certain oxidation products of these substances from its food it completely excludes the isomeric and very similar lycopene which has been found in the faeces in considerable quantity after tomato feeding. The mechanism of this selective absorption of one isomer only is quite obscure.

2. Adsorption of the carotenoids of several colostrum fats gave no indication of the presence of lycopene but the adsorption columns contained two

small zones above that of β-carotene due to traces of pigments having absorption maxima at 452 and 425 mμ (petroleum ether b.p. 70–80°). These may be due to oxidation products of β-carotene.

Our best thanks are due to members of the Dairy Husbandry Department of the N.I.R.D. for help in this work and especially to Mr K. L. Blaxter.

REFERENCES

(1) PALMER & ECKLES (1914). *J. biol. Chem.* **17**, 191.
(2) KUHN & LEDERER (1931). *Hoppe-Seyl. Z.* **200**, 246.
(3) KUHN & BROCKMANN (1933). *Ber. dtsch. chem. Ges.* **66** B, 407.
(4) GILLAM & HEILBRON (1935). *Biochem. J.* **29**, 834.
(5) STRAIN (1935). *J. biol. Chem.* **111**, 85.
(6) GILLAM & EL RIDI (1937). *Biochem. J.* **31**, 251.
(7) STRAIN (1939). *J. biol. Chem.* **127**, 191.
(8) MEIGS (1938). *Rep. Bur. Dairy Ind. U.S. Dep. Agric.* p. 22.
(9) QUACKENBUSH, STEENBOCK & PETERSON (1938). *J. Amer. chem. Soc.* **60**, 2937.
(10) VAN WIJNGAARDEN (1935). *Carotine, vitamine A en vitamine C in koemelk.* Thesis, Univ. Utrecht (N. V. Drukkerij p. den Boer, Utrecht).
(11) WILLSTAEDT & WITH (1938). *Hoppe-Seyl. Z.* **253**, 133.
(12) WITH & FRIDERICHSEN (1939). *Ugeskr. Laeg.* **101**. 915.
(13) GILLAM (1934). *Biochem. J.* **28**, 79.
(14) KUHN & GRUNDMANN (1932). *Ber. dtsch. chem. Ges.* **65** B, 1880.
(15) CASTLE, GILLAM, HEILBRON & THOMPSON (1934). *Biochem. J.* **28**, 1702.
(16) GILLAM & EL RIDI (1936). *Biochem. J.* **30**, 1735.
(17) CARTER & GILLAM (1939). *Biochem. J.* **33**, 1325.
(18) ZECHMEISTER & TUZSON (1938). *Biochem. J.* **32**, 1305.
(19) EULER, KARRER & WALKER (1932). *Helv. chim. Acta*, **15**, 1507.

The values of 2200 and 1300 used throughout this paper are the intensities of adsorption, for carotene and vitamin A respectively, in chloroform solution. For the meaning of the term and the effect of solvent on the intensity of adsorption, cf. Gillam, *Biochem. J.* **29**, 1831 (1935) and Gillam & El Ridi, *Biochem. J.* **32**, 820 (1938).

(*MS. received for publication* 20 *May* 1940)

576.809.515

264. THE EFFECT OF VARIATIONS IN THE FAT PERCENTAGE AND IN THE REACTION (*p*H) OF MILK MEDIA ON THE HEAT RESISTANCE OF CERTAIN MILK BACTERIA

By AGNES A. NICHOLS
The Hannah Dairy Research Institute, Kirkhill, Ayr, Scotland

(With 2 Figures)

PART I. THE HEAT RESISTANCE OF AEROBIC SPOREFORMERS

Introduction

The difficulties of investigations into the heat resistance of bacteria are intensified when the organisms under consideration are in the form of spores. In work of this type many factors are involved, and when any one of these is under study the remainder must be rigidly controlled and standardized. The whole problem, too, is often further confused by the dormancy or delay in germination of the spores themselves after they have been heated under experimental conditions, so that the numbers of surviving spores are often masked. These difficulties will be better appreciated by reference to some of the more important publications which have already appeared on the subject.

Bigelow & Esty(1) were probably among the earliest to investigate extensively the heat resistance of spores. In studies with a thermophilic aerobic sporeformer they showed the importance of hydrogen-ion concentration and of the concentration of spores. Much work on *Cl. botulinum* and allied anaerobes followed, and it was demonstrated(2) that the media in which the spores developed, the age of the spores, their concentration and the *p*H and salt concentration of the substrate all affected the thermal death-rate.

Burke(3) showed that individual spores of *Cl. botulinum* might vary in the time required for germination and that they might germinate quickly or lie dormant for long periods after heating. It was also shown that cultures heated for periods up to a given temperature all grew uniformly when incubated under favourable conditions, but if heated for longer periods, when single tubes were used, the results would be irregular and inconsistent. Thus spore suspensions in a single-tube series that had been treated for a period far in excess of those in which uniform growth had been observed might contain viable organisms, even though several intermediate tubes were found to be sterile. These so-called "skips" were at first thought to be due to faulty technique but were later accounted for on the grounds of variation in the resistance of individual spores. Esty & Williams(4) therefore suggested a multiple tube method for

recording the heat resistance, and stated that by using thirty tubes of the same suspension heated for at least four different times at a fixed temperature a logarithm chart could be prepared from which it was possible to predict the resistance of the spores when one factor was varied. They demonstrated the usefulness of such a method in the control of *Cl. botulinum*. The work on the dormancy of this organism was extended to show that "skips" did not depend only on heat treatment, but that delayed germination could mask the survival figures and that sudden changes in temperature or agitation accelerated germination after heat treatment. Dickson *et al.* (5), in pointing this out, also stated that the heat resistance was greatest in broth which was covered with a thin layer of oil.

The heat resistance of *B. subtilis* and other common aerobic sporeformers, including *B. cereus*, *B. mycoides* and *B. mesentericus*, was investigated by Williams (6). He found that the resistance of spores varied according to the kind of media on which they were produced and with the temperature of cultivation, but that dormancy was practically non-existent, since only four out of a total of 3442 tubes which he tested failed to develop until after the eighth day of incubation. Sommer (7) aimed at establishing a standard procedure for the mass production of heat-resistant *Cl. botulinum* spores, and having developed resistant spores he sought to determine a technique for keeping them uniformly resistant. The results of several other workers were reviewed by him, and of these the studies of Magoon (8,9) should be particularly noted. Magoon investigated several different types of sporeformers, amongst which were included the aerobic sporeforming bacteria. He concluded that many factors, some of them unknown, affected the development of heat-resistant spores, and that there was no definite regularity of behaviour for different lots of spores under the methods of storage which he investigated. Williams (10) has attempted more recently to increase the heat resistance of spores by selecting single cells of three anaerobic and three aerobic sporeformers. He was, however, only able to show a significant increase with *B. mycoides*. He also noticed the "skip-stop" phenomena with one of the anaerobic sporeformers which he examined.

Morrison & Rettger (11) found that the heat resistance and dormancy of two strains of bacteria were variable when the spores were heated in water and subcultured in plain nutrient broth. However, when the spores were heated in evaporated milk with or without subculturing the results were regular and no dormancy was recorded, which suggested that unfavourable environment was the controlling factor. These authors further examined (12) the relationship of spore germination to environment, and found that some organisms, e.g. *B. subtilis* and probably *B. cereus* and *B. vulgatus*, displayed no delayed germination, but *B. megatherium* behaved with inexplicable idiosyncrasies in certain media, and the strains which they had isolated from milk spoilage showed marked peculiarities in requirements for germination in different media.

576.809.515

264. THE EFFECT OF VARIATIONS IN THE FAT PERCENTAGE AND IN THE REACTION (*p*H) OF MILK MEDIA ON THE HEAT RESISTANCE OF CERTAIN MILK BACTERIA

By AGNES A. NICHOLS
The Hannah Dairy Research Institute, Kirkhill, Ayr, Scotland

(With 2 Figures)

PART I. THE HEAT RESISTANCE OF AEROBIC SPOREFORMERS

Introduction

The difficulties of investigations into the heat resistance of bacteria are intensified when the organisms under consideration are in the form of spores. In work of this type many factors are involved, and when any one of these is under study the remainder must be rigidly controlled and standardized. The whole problem, too, is often further confused by the dormancy or delay in germination of the spores themselves after they have been heated under experimental conditions, so that the numbers of surviving spores are often masked. These difficulties will be better appreciated by reference to some of the more important publications which have already appeared on the subject.

Bigelow & Esty (1) were probably among the earliest to investigate extensively the heat resistance of spores. In studies with a thermophilic aerobic sporeformer they showed the importance of hydrogen-ion concentration and of the concentration of spores. Much work on *Cl. botulinum* and allied anaerobes followed, and it was demonstrated (2) that the media in which the spores developed, the age of the spores, their concentration and the *p*H and salt concentration of the substrate all affected the thermal death-rate.

Burke (3) showed that individual spores of *Cl. botulinum* might vary in the time required for germination and that they might germinate quickly or lie dormant for long periods after heating. It was also shown that cultures heated for periods up to a given temperature all grew uniformly when incubated under favourable conditions, but if heated for longer periods, when single tubes were used, the results would be irregular and inconsistent. Thus spore suspensions in a single-tube series that had been treated for a period far in excess of those in which uniform growth had been observed might contain viable organisms, even though several intermediate tubes were found to be sterile. These so-called "skips" were at first thought to be due to faulty technique but were later accounted for on the grounds of variation in the resistance of individual spores. Esty & Williams (4) therefore suggested a multiple tube method for

recording the heat resistance, and stated that by using thirty tubes of the same suspension heated for at least four different times at a fixed temperature a logarithm chart could be prepared from which it was possible to predict the resistance of the spores when one factor was varied. They demonstrated the usefulness of such a method in the control of *Cl. botulinum*. The work on the dormancy of this organism was extended to show that "skips" did not depend only on heat treatment, but that delayed germination could mask the survival figures and that sudden changes in temperature or agitation accelerated germination after heat treatment. Dickson *et al.* (5), in pointing this out, also stated that the heat resistance was greatest in broth which was covered with a thin layer of oil.

The heat resistance of *B. subtilis* and other common aerobic sporeformers, including *B. cereus*, *B. mycoides* and *B. mesentericus*, was investigated by Williams (6). He found that the resistance of spores varied according to the kind of media on which they were produced and with the temperature of cultivation, but that dormancy was practically non-existent, since only four out of a total of 3442 tubes which he tested failed to develop until after the eighth day of incubation. Sommer (7) aimed at establishing a standard procedure for the mass production of heat-resistant *Cl. botulinum* spores, and having developed resistant spores he sought to determine a technique for keeping them uniformly resistant. The results of several other workers were reviewed by him, and of these the studies of Magoon (8,9) should be particularly noted. Magoon investigated several different types of sporeformers, amongst which were included the aerobic sporeforming bacteria. He concluded that many factors, some of them unknown, affected the development of heat-resistant spores, and that there was no definite regularity of behaviour for different lots of spores under the methods of storage which he investigated. Williams (10) has attempted more recently to increase the heat resistance of spores by selecting single cells of three anaerobic and three aerobic sporeformers. He was, however, only able to show a significant increase with *B. mycoides*. He also noticed the "skip-stop" phenomena with one of the anaerobic sporeformers which he examined.

Morrison & Rettger (11) found that the heat resistance and dormancy of two strains of bacteria were variable when the spores were heated in water and subcultured in plain nutrient broth. However, when the spores were heated in evaporated milk with or without subculturing the results were regular and no dormancy was recorded, which suggested that unfavourable environment was the controlling factor. These authors further examined (12) the relationship of spore germination to environment, and found that some organisms, e.g. *B. subtilis* and probably *B. cereus* and *B. vulgatus*, displayed no delayed germination, but *B. megatherium* behaved with inexplicable idiosyncrasies in certain media, and the strains which they had isolated from milk spoilage showed marked peculiarities in requirements for germination in different media.

Curran (13) investigated the influence of environmental factors on the thermal resistance of spores of *B. cereus* isolated from defective evaporated milk and concluded that the resistance of spores is not a fixed property and that the direction and extent of the change is largely determined by a number of physical and chemical forces operating outside the cell, as, for example, temperature of storage and dryness.

In view of the above facts it was felt that, before attempting any general study of the factors affecting the heat resistance of the sporeformers isolated from canned milk and cream, it would be advisable to obtain some approximate information as to their heat resistance, since certain peculiarities of behaviour (for instance, dormancy) might then become apparent which would necessitate modifications in the experimental technique.

Experimental

Substrate container for heat-resistance tests

The container in which inoculated substrate is to be heated presents certain problems of technique. The difficulties connected with the choice of a container are particularly evident at the high temperatures which are employed for the aerobic sporeformers and when milk or cream is used as the substrate. The points of importance in selecting a suitable container are: (i) that it should be constructed so that the time necessary for the contents to reach the same temperature as the liquid in the surrounding bath is reduced to a minimum; (ii) that few breakages should occur during operation; (iii) that it should be easy to fill with substrate, or, if the substrate has been sterilized inside the container, it should be simple to inoculate it quickly; and (iv) that the examination for spore germination after heating should be simple and the possibilities of contamination of the substrate when transferred to other media be negligible.

A variety of containers has been suggested and the following have been tried out:

(*a*) *Glass capillary tubes.* In these the temperature lag in heating the substrate was obviously very small, but in practice this advantage was outweighed by certain other factors. It was found, for example, that large numbers of capillary tubes broke or leaked when introduced into the hot bath, and that even if the capillary tubes survived heating the transfer of the heated material into media for incubation without introducing outside contamination was difficult. This problem was particularly evident when the milk media clotted at the higher temperatures. In addition, thick cream could not readily be drawn up into the capillaries.

(*b*) *Glass ampoules or bulbs.* Ampoules of glass sufficiently resistant to withstand autoclaving and heating without breakdown were not only difficult to seal quickly but were also difficult to break open before transfer of the

contents into media. Small ampoules were desirable so that the heating lag would be short, and consequently the ampoules were narrow-necked and only Pasteur pipettes could be used for filling them. It was again found particularly difficult to do this when the substrate was cream.

(*c*) *Glass Pasteur pipettes* were used as follows. The inoculated substrate was drawn up into the pipette which was then sealed at both ends. If pipettes were used which had a wide tip, cream could be drawn up readily by this method. These containers have thicker glass walls than the two already mentioned, and hence there would be a greater temperature lag in the heating of the substrate. It was found, however, that, although the filling was simplified, the breakages, which amounted to about 30 %, were so high that this container could not be employed satisfactorily.

If only a few heat-resistant tests had been required any of the above containers could probably have been used successfully, but as large numbers of tests were entailed a method which involved frequent breakages was not practical. A further method was therefore explored in which use was made of special test tubes.

(*d*) *Test tubes.* After thorough shaking, 1 ml. amounts of the inoculated substrate were transferred by pipetting into small rimless test tubes, which were made of hard resistant glass. The tubes were 3 in. long and had an external diameter of $\frac{7}{16}$ in., and an internal diameter of $\frac{3}{8}$ in. The tubes were previously sterilized and plugged with cotton-wool, but after filling were tightly corked by rubber bungs, which had been wrapped separately in grease-proof paper and sterilized in the autoclave in Kilner jars before use. The rubber bung usually extended down into the tube for at least $\frac{1}{2}$ in. The tubes containing substrate were placed in a small metal clamp which was screwed down so that the bung would continue to fit tightly even when exposed to high temperatures (Howat & Wright(14)).

Usually a large bulk of substrate was inoculated with the test culture, except in the trial tests on the sporeformers when the substrate contained in the tubes was inoculated directly from the culture.

These small tubes fulfilled the requirements for a suitable container, since breakages were not very great. Such breakages as did occur were mainly with new batches of glassware or were caused before heating when the tubes were being stoppered. Large numbers of tubes could be filled quickly with equal volumes of substrate, so that if the substrate were inoculated in bulk the number of organisms in each tube would be uniform. After heating, the tubes could be incubated directly for observations on the survival of bacteria, or the whole of the contents could be transferred very readily into tubes of other media either by direct pouring or by pipetting. It should be noted that in these studies litmus milk was the only medium used for reinoculation purposes, and that in the many tubes transferred in this way contamination occurred very infrequently. Regarding heat transfer, this was obviously less efficient than with capillary tubes, but uniform tubes were used throughout so that the

lag in attaining the maximum temperature was a constant factor. In the studies with non-sporeformers, 2 min. were allowed to lapse before the recording of the time of heating began. With the sporeformers, however, because of the cooling effect on the glycerine when the cold tubes were introduced into the bath, the tubes were held for 2 min. in boiling water before being transferred to the bath, and the time was recorded from the moment of their immersion in the glycerine.

General heat-resistance trials with aerobic sporeformers

In order to obtain general information on the heat resistance of aerobic sporeformers cultures of these organisms were grown on milk agar slants for 2 days at 37° C. and held at room temperature for 3 days before being inoculated directly into skimmed milk, the latter having been previously sterilized in the tube containers just described. The tubes were subjected to temperatures between 70 and 120° C. for periods of 20 min., the latter being in keeping with the period of heating used in commercial canning. After heating, the tubes were cooled and incubated at 37° C. In most instances the survival of the organisms in the tubes was readily apparent on incubation owing to the characteristic digestion of milk. Microscopic examination was used as a confirmatory test. Where there was any doubt about the survival of the organisms, or if it appeared that none had withstood heating, the contents of the tube were transferred into litmus milk media and incubated for a minimum of 2 weeks but more often for 1 month. Usually the heat exposure tests were carried out in duplicate and frequently in triplicate. The results are shown in table 1.

A further general trial of heat resistance was carried out with some of the above strains and also with certain additional cultures, the number of strains examined totalling nearly 40. In this further trial, however, one inoculated tube of skimmed milk, another of whole milk and a third of cream (containing 23 % butterfat) were heated at each temperature. As before, the tube contents were ultimately transferred into litmus milk and the survival in cream was read in nearly all instances by this method, since the consistency of cream made direct reading of growth difficult in the early stages. The results are shown in Table 2.

From a study of Tables 1 and 2 it will be seen that the cultures showing the greatest heat resistance were strains of *B. subtilis* and that the variations within strains of the same organism were considerable. Thus, while the majority withstood the heat of 120° C. for 20 min., other strains (for example, no. 7), which had also been isolated from processed milk, did not appear to have a great tolerance to heat. When the same culture was examined more than once absolute checks were not obtainable, but even allowing for the large temperature interval between the tests this lack of agreement was less in some cases than might have been expected from the review of previous work,

Table 1. *Resistance of sporeformers to heat in skimmed milk* (20 *min. exposures*)

Temp. of exposure °C.	Species: *B. licheniformis* Culture no. 29	91	92	93	94	95	*B. cereus* 40	*B. brevis* 20	*B. circulans* 49	67	
70	.	.	.	.	.	.	.	.	+ +	.	
80	.	.	.	.	.	.	.	.	+ −		+ +
90	.	+ + +	+ + +	+ + +	+ + +	+ + −	+ +	.	.	+ +	+ +
100	+ −	+ + +	+ −	− − −	+ + −	− −	+ +	+ +	+ −	+ +	+ +
105	.	+ +	− −	− −	− −	+ −	+ +	+ +	.		+ +
110	+ −	+ + +	− − −	− −	− − −	+ + −	+ −	+ −	.	− −	+ −
115	+ +	− −	+ +	+ −	− −	− − −	− −	.	.	.	
120	− −	.	.	.	.	.	−	.	.	.	

	Species: *B. subtilis* Culture no. 1	2	4	7	13	24	26	27	62	79	80	87	88	89	90	77	78
70	.	.	.	.	+ +	+ +	.	.	.	.	.	.	.	.	.	.	.
80	.	.	.	.	+ +	+ +	.	.	.	.	.	.	.	.	.	.	.
90	.	.	.	.	+ +	+ +	.	.	.	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ +	+ +
100	+	+	+	+ +	+ +	+ +	.	.	.	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ +	+ +
105	+ +	+ +	+ +	+ +	+ +	.	.	.	.	.	.	+ +	+ +	+ +	+ +	+ +	+ +
110	+ +	+ +	+ −	−	+ +	+ +	.	+ +	+ +	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ +	+ +
115	+ +	+ +	+ −	− −	.	+ +	+ +	+ +	+ +	+ +	− −	+ −	− −	− −	− −	+ +	+ +
120	+ +	+ −	− −	.	.	+ +	+ −	+ +	− −	+ −	− −	.	.	.	.	− −	− −
125	.	.	.	.	.	.	− −	− −	− −	− −	− −	.	.	.	.	.	.

Table 2. *Resistance of sporeformers to heat in skimmed milk, milk and cream (23 % butterfat; 20 min. exposures)*

Temp. of exposure °C.	Substrate	*B. brevis*		*B. circulans*	*B. licheniformis*		*B. subtilis*														
		20	22	49	29	54	1	2	3	4	5	6	7	26	27	27	54	55	57	58	59	60	62
80	Skimmed milk	.	.	+	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.
	Milk	.	.	+	+	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.
	Cream	.	.	+	+	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.
90	Skimmed milk	+	+	+	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.
	Milk	+	+	+	+	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.
	Cream	+	+	+	+	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.
100	Skimmed milk	+	+	+	.	+	+	+	+	+	+	+	+	+	.	+	+	+	+	+	+	+	+
	Milk	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Cream	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+	+	−	+
110	Skimmed milk	−	+	−	.	+	+	+	−	+	+	−	−	.	.	+	+	+	+	+	−	+	.
	Milk	−	+	−	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+
	Cream	−	+	−	+	+	+	+	−	+	+	−	−	+	+	+	+	+	+	+	−	+	+
115	Skimmed milk	−	+	.	.	−	−	−	−	+	−	−	−	+	.	+	−	+	−	−	−	−	.
	Milk	−	−	.	.	−	+	+	−	+	−	−	−	+	−	+	−	−	−	−	−	−	+
	Cream	−	−	.	.	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−	−
120	Skimmed milk	−	−	.	.	−	−	−	−	+	+	−	−	.	+	−	−	−	−	+	−	.	−
	Milk	−	−	.	.	−	−	−	−	+	−	−	−	−	+	+	−	−	−	−	−	.	+
	Cream	−	−	.	.	−	+	+	−	+	−	−	−	.	−	+	−	−	−	+	−	.	−
125	Skimmed milk	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	−	−	−	+	−	.	.
	Milk	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	−	−	−	−	−	.	.
	Cream	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	−	−	−	−	−	.	.

since many of the precautions which have been shown necessary for uniformity were not taken in this general trial.

It will be noted that "skips" occurred in some tests, especially among the *B. licheniformis* species (Table 1). When cream, milk and separated milk were used as substrates there was no indication that the bacteria in any one medium showed a greater heat resistance than in either of the others. Dormancy was not observed, although in some tubes the spores took 1 or 2 days longer than normal to germinate. The survival of the spores was always evident within 1 week's incubation and more often after 3–4 days.

Special studies of the heat resistance of aerobic sporeformers

In view of the commercial importance of the subject in the production of evaporated milk and canned cream, it was decided to investigate in greater detail the heat resistance of some of the aerobic sporeformers, particularly in relation to the *p*H and butterfat content of the substrate. For this purpose strains of *B. subtilis* were selected because it appeared both from the numbers isolated from cans and from the general trials of heat tolerance that organisms of this species were the most likely to survive commercial canning. Nos. 6, 27 and 62 were selected, since they were known to be active in sterilized cream spoilage (15). These strains were also representative of the variation in heat tolerance of the strains of *B. subtilis* which were isolated from canned products, no. 6 having a less pronounced heat resistance than that of the other two cultures.

Preparation of spore suspensions. Spore suspensions were prepared as follows: The cultures were inoculated on to milk-agar slants prepared in large flat-sided medicine bottles. The slants were about 20 sq. in. in extent, and they were incubated for 48 hr. at 37° C. after inoculation. The cultures were then washed off with sterile distilled water and incubated for a further 24 hr. at 37° C. to increase sporulation. The water suspension was well shaken up with glass beads to break up the clumps, and filtered through a filter paper containing a heavy layer of sterilized absorbent cotton-wool, which had been previously sterilized. The filtering process removed any remaining clumps, and absence of clumps of spores in the final suspension was confirmed by microscopic examination. The suspension was heated to 190° F. for 10 min. in water to destroy any vegetative forms, and it was then cooled quickly and placed in a small screw-capped bottle in a refrigerator at 40° F. until required. Since very good checks were obtained when several plates of the same dilution were counted and since dormancy did not appear to occur, it was clear that the counting of the number of spores by specialized methods was unnecessary, and the number of spores present in the suspension was therefore determined by plate count on milk agar. It may be noted that when plate counts were made of the sporeformers sterile blotting paper and glycerine were placed inside the plates after they had been poured and inverted to absorb the water of condensation and prevent the colonies from spreading.

(i) *The effect of the reaction (pH) of the substrate on the destruction of* B. subtilis *by heat.*

Cream of 23·5 % butterfat content was used as the substrate. By the additions of small quantities of *N*/10 NaOH or *N*/10 HCl, a titration curve was obtained for the particular cream, and by using the quantities indicated by the curve it was possible by adding sterile alkali and acid to adjust the sterilized substrate to different *p*H values. After the initial adjustment in *p*H had been made the subsamples of cream were thoroughly shaken and were not used for heat-resistance tests until at least 24 hr. had elapsed, i.e. when the *p*H had reached equilibrium. The *p*H was then determined by the quinhydrone electrode, and equal volumes of the spore suspension were added to each of the subsamples.

The effect of changes in the reaction of the substrate on the destruction of *B. subtilis* strains nos. 6 and 62 was investigated. Both cultures were tested in four subsamples of cream substrate. The *p*H and the plate counts of the subsamples which were tested immediately before the heat-resistance tests commenced, are given below.

Culture no.	Subsample no.	*p*H of subsample	Plate count per ml. of subsample	Temperature at which tubes were heated for resistance tests ° C.
6	1	7·0	1,700,000	116
	2	6·7	1,610,000	
	3	6·3	1,540,000	
	4	6·1	1,520,000	
	5	5·95	1,440,000	
62	1	7·25	40,000	120
	2	7·0	45,000	
	3	6·6	51,000	
	4	6·1	38,000	

It will be seen that the plate counts of each of the four subsamples in both experiments were very similar. The range of *p*H values chosen for the experiments was relatively small, but covered that found in commercial canning. After the substrate had been inoculated 1 ml. amounts were transferred to the tube containers already described. If there was any delay between the filling of the tubes and the heat treatment the tubes were cooled down in iced water and retained at 40° F. The temperature of heating was kept constant in both experiments, and the time of exposure was varied from 16 to 60 min. From three to five different periods of heat exposure were given to each subsample, twenty-five tubes being used for each different time of exposure. As before the tubes were first held in boiling water for 2 min. before they were placed in the bath, and they were cooled in cold water after heating and then incubated at 37° C. for 1 month. The numbers of tubes in which growth occurred after heating was recorded and has been plotted in Figs. 1 and 2 as the percentage of tubes in which spores survived (i.e. the survival percentage) against the length of time for which heating was carried out.

The results show clearly that as the period of exposure to heat increases the survival percentage decreases. It will be seen, however, that there is a lack of consistency in the results, and that over the restricted range of *p*H examined it is impossible to define the effect of change in the *p*H on the heat resistance of the spores in cream. It appears from the general examination of the results that increases or decreases in the values on either side of a *p*H of about 6·6–6·8 may tend to decrease the resistance of the spores to heat.

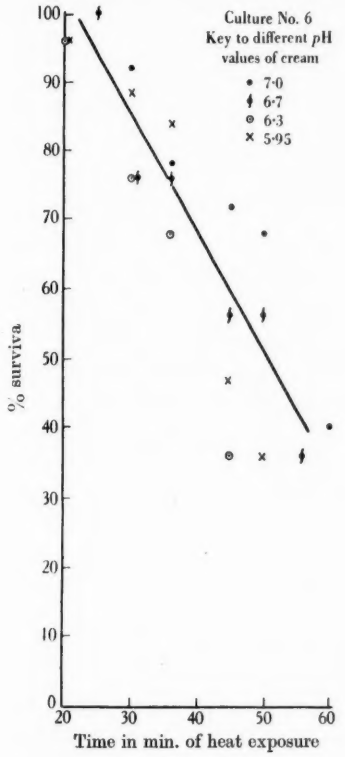

Fig. 1.

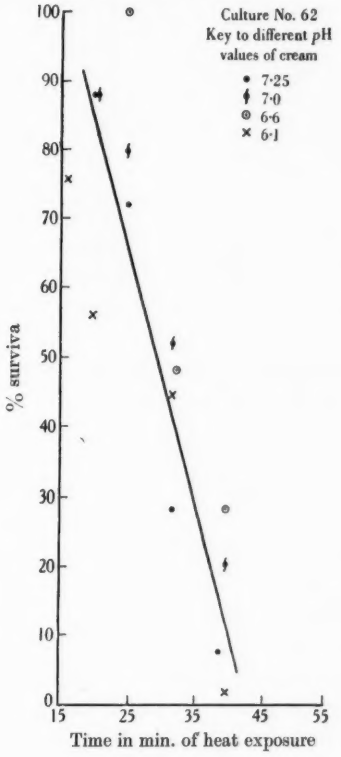

Fig. 2.

(ii) *The effect of increased butterfat percentage on the destruction of* B. subtilis *by heat.*

(*a*) *By survival percentage method.* The effect of increasing the butterfat percentage on the heat resistance of *B. subtilis* in milk was investigated by two methods. The first method was similar to that just described. The percentage of tubes in which spores survived in the different subsamples, which has been based on the results from about twenty-five tubes, is given in Table 3. The plate counts of the subsamples, together with the cultures examined and the different temperatures at which heat-resistance experiments were carried out, are also noted.

Table 3. *Survival percentage*

Time retained at temperature min.	Skimmed milk	Milk 3·5 % butterfat	Cream 10 % butterfat	Cream 23 % butterfat
	A. Culture no. 6 heated at 110° C.			
20	97	100	100	93
22	82	100	100	100
25	64	100	96	93
27	61	90	83	70
30	36	79	73	50
35	3	35	30	40
38	—	17	20	—
40	—	40	15	7
50	5	—	—	—
	B. Culture no. 62 heated at 115° C.			
7½	100	100	—	100
10	100	100	—	—
12½	100	—	100	—
15	96	100	100	100
20	88	100	68	100
22½	88	92	96	64
25	88	100	89	7
27½	—	76	84	10
30	96	88	88	0
32½	—	—	88	0
35	—	88	80	—
37½	—	80	—	—
40	—	80	—	—
	C. Culture no. 27 heated at 120° C.			
12	100	—	100	100
14	96	100	—	—
16	100	100	88	88
18	92	92	68	—
20	40	56	88	92
22	72	68	84	—
25	48	40	52	80
30	48	76	68	40
35	—	—	76	36
40	—	—	88	12
50	—	—	88	—
	D. Culture no. 6 heated at 116° C.			
18	92	—	—	—
21	100	88	76	88
24	80	64	88	84
27	92	64	72	80
30	—	88	76	68
33	96	80	80	80
36	—	72	64	—
39	88	60	61	72
41	48	—	—	—
42	—	48	60	48
45	32	72	—	—
48	—	63	56	36

Table 3 (*continued*)

E. Culture no. 6 heated at 110° C.

Time retained at temperature min.	Skimmed milk	Time retained at temperature min.	Milk 3·5 % butterfat	Cream 11 % butterfat
20	96	25	100	92
23	72	26½	100	—
26	76	28	92	84
27½	92	29½	100	78
29	84	31	92	72
30½	76	34	72	92
32	52	35½	96	—
35	72	37	100	76
38	56	40	84	80
41	44	43	96	60
44	72	46	88	—
47	78	49	52	—

Plate count per ml. of substrate

	Skimmed milk	Milk 3·5 % butterfat	Cream 10 % butterfat	Cream 23 % butterfat
A	950,000	1,225,000	1,190,000	1,270,000
B	180,000	—	160,000	167,000
C	283,000	806,000	297,000	293,000
D	2,110,000	2,880,000	1,950,000	1,570,000
E	2,160,000	—	—	3,200,000

It will be seen that there is little uniformity in the results of the tests, a fact which again shows the difficulties in dealing with sporeformers in heat-resistance experiments. However, the results do indicate that the raising of the percentage of butterfat does not increase the heat resistance of the organisms under test. In fact, a general examination of the results indicates that the reverse is more probable.

(*b*) *By a plate-count method.* A further attempt was made to determine whether an increase in butterfat content changes the heat resistance of sporeformers. Substrates were prepared as before, having different butterfat contents. They were prepared from Grade A (T.T.) milk, and they were not sterilized. Spore suspensions of culture 62 were added (as in the previous experiments) in such a way that the count would be as nearly as possible the same in each subsample. Plate counts were made on the substrates before and after adding the spores, and approximately 4 ml. amounts of the subsamples, both before and after the spores had been added, were set up in the special tube containers for heat-resistance tests. The tubes containing the inoculated substrate were held at different temperatures for varying periods of time, and immediately thereafter plate counts were made. The results are given in Table 4.

It will be seen that the plate counts of the uninoculated subsamples before heating were low, and that after heating no bacteria could be detected on the 1 ml. plates. All samples were retained in iced water after inoculation if there was any delay before heating so that no multiplication of bacteria could have occurred. It is clear, therefore, that the lack of absolute sterility of the subsamples did not appreciably alter the results.

Table 4

		Heat treatment			Plate count at 37° C.		
A. No. 62		Temp. °C.	Time min.	Skimmed milk	Milk 2·95 % butterfat	Cream 12·5 % butterfat	Cream 24·5 % butterfat
I. Uninoculated substrate	Unheated	Control		6,200	6,000	3,700	6,000
	Heated	100	20	0	0	0	0
Inoculated substrate	Unheated	Control		4,300,000	5,500,000	5,500,000	7,500,000
	Heated	100	20	3,200,000	1,970,000	4,000,000	3,680,000
		110	20	1,200,000	1,152,000	1,440,000	1,616,000
		115	20	0	30	30	30
II. Uninoculated substrate	Unheated	As above					
	Heated						
Inoculated substrate	Unheated	Control		1,080,000	1,270,000	840,000	810,000
	Heated	110	20	1,180,000	1,220,000	1,320,000	1,430,000
		110	30	148,000	142,000	314,000	580,000
		115	10	138,000	117,000	—	222,000
		115	20	42,000	63,000	76,000	82,000
		115	15	2,800	2,780	2,090	2,060

		Heat treatment			Plate count at 37° C.			
B. No. 62		Temp. °C.	Time min.	Skimmed milk	Milk 3 % butterfat	Cream 12 % butterfat	Cream 22 % butterfat	Cream 35 % butterfat
I. Uninoculated substrate	Unheated	Control		1,500	2,000	1,400	600	1,200
	Heated	115	10	0	0	0	0	0
Inoculated substrate	Unheated	Control		3,000,000	2,000,000	2,300,000	4,200,000	4,000,000
	Heated	115	10	77,000	51,000	73,000	35,000	79,000
		115	15	1,140	1,290	4,000	1,480	4,000
		115	20	30	480	30	130	60
		110	40	0	0	0	0	50
II. Uninoculated substrate	Unheated	As above						
	Heated							
Inoculated substrate	Unheated	Control		2,320,000	2,020,000	1,690,000	2,100,000	2,020,000
	Heated	115	6	1,732,000	1,602,000	1,732,000	1,678,000	1,856,000
		115	10	77,000	64,000	63,000	53,000	54,000
		113	12	640,000	570,000	750,000	660,000	870,000
		110	30	480,000	890,000	880,000	460,000	210,000

The plate counts indicate more definitely than did the survival percentages that an increase in the butterfat content of the subsamples does not increase the resistance of the spores to heat.

It seems that the spores of these strains of *B. subtilis* were so resistant to heat that the minor changes which were made in *p*H and in the composition of the substrates in the present studies were not sufficient to affect the heat resistance.

PART II. THE HEAT RESISTANCE OF CERTAIN NON-SPOREFORMERS

The previous section has dealt with the effects of alterna.ives in the *p*H and butterfat content of the substrate on the heat resistance of aerobic sporeformers. The present section deals with certain non-sporeforming organisms which have been isolated from canned milk products. In an earlier publication the author (16) has shown that the destruction of milk bacteria by pasteurization is affected by changes in the *p*H of the milk. In addition to the *p*H of the substrate, the composition of the medium itself has been claimed (1) to have an important effect on the heat resistance of certain non-sporeformers. In particular, Brown & Peiser (17) claim to have demonstrated that a high butterfat content confers a definite protective action on certain lactic acid bacteria. Their conclusions were based on comparisons of the thermal death-points (using 10 min. exposures to different temperatures) of the organisms in cream of 25 % butterfat content, in whole milk, in separated milk, in whey and in bouillon. Their results showed that the temperature necessary to destroy all bacteria was lowest in broth, was from 2 to 4° C. higher in whey, from 4 to 6° C. higher in separated milk, about 8° C. higher in whole milk and from 12 to 14° C. higher in cream. The milk media were all made from the same sample of milk and were sterilized intermittently in flowing steam. Brown & Peiser attribute this marked increase in the heat resistance with the different milk media solely to the protective action of the butterfat.

It has already been seen that no such protective action was found by the present author in the study of aerobic sporeformers. It was felt desirable to re-investigate the findings of Brown & Peiser by studying the effect of increased butterfat percentage on the heat destruction of certain selected non-sporeforming bacteria.

EXPERIMENTAL

The substrates were prepared by separating fresh mixed milk. The butterfat contents varied slightly with the different batches of media but fell within the following ranges:

Substrate	Butterfat content %
Separated milk	0·1
Whole milk	3·8–4
Cream	10–12
Cream	22–25

The *p*H of the above media was determined by the quinhydrone electrode before and after sterilizing. From the examples given below it will be seen that the differences before sterilization were not great enough to justify further adjustment, and that after sterilization the differences did not exceed 0·2 *p*H. Hence, in the range of media used, the *p*H varied only slightly, and since the *p*H of the media rose as the fat percentage increased, any change in the *p*H of the cream would tend to operate in favour of slightly greater heat resistance.

	Before sterilization	After sterilization	Before sterilization	After sterilization
Separated	6·58	6·34	6·35	6·00
Whole milk	6·61	6·39	6·32	6·07
Cream (24 % fat)	6·68	6·51	6·61	6·11

All the media were homogenized, since this was essential for the cream samples and was in any event typical of the conditions which would prevail in commercial manufacture. They were sterilized in bulk by steaming on three successive days at 100° C. for 30 min.

Four non-sporeforming cultures, nos. 15, 17, 31 and 33, which were species of the genus *Escherichia* (i.e. were similar to some of the organisms used by Brown & Peiser) and which produced an acid and gassy clot in litmus milk, were grown separately on several standard agar slants. In addition two cultures, no. 63 of *Str. thermophilus* and no. 106 of *Str. durans*, both of which produced acid coagulation in milk, were used. A suspension was prepared of each of the six cultures examined by washing the growth, after 2 days' incubation at 37° C., off the slants with sterile distilled water. The suspension was shaken thoroughly in a screw-capped bottle in which glass beads were used to assist in breaking up the clumps, and was filtered through sterile cotton-wool and a filter paper to remove any remaining clumps. An equal volume of the suspension was added to 200 ml. of skimmed milk, milk and cream of 10 % butterfat. Cream substrate containing 23 % butterfat was difficult to pipette if it was not used almost immediately, since it underwent age thickening and it was only used in this experiment with cultures nos. 63 and 106. After thorough shaking the samples were plated on milk agar and 1 ml. amounts of the inoculated substrates were filled into small resistant glass test-tubes and heated in triplicate or quadruplicate in a thermostatically controlled gylcerine bath (see Part I for a discussion on the container and method of heating adopted). Three cultures, nos. 33, 63 and 106, were exposed for 10 min. to a number of different temperatures, while the remaining other three cultures were held at 60° C. for different lengths of time. In each experiment 2 min. were allowed to elapse before recording the commencement of heating. After heating, the tubes were immediately cooled in cold water, and they were then incubated at 37° C. The survival of any bacteria was judged from inspection of the media after incubation.

The results are given in Table 5 A, B. The plate counts of the original inoculated substrates prior to heating were very similar, and it is therefore

Table 5. ***Bacterial resistance to heat in milk substrates of increasing butterfat percentage***

(A) Fixed time, variable temperature

(B) Fixed temperature, variable time

Table 5. *Bacterial resistance to heat in milk substrates of increasing butterfat percentage*

(A) Fixed time, variable temperature

Plate count of inoculated substrate before heating ...	Substrate	Culture no. 33	Substrate	Culture no. 63	Substrate	Culture no. 106
	Skimmed milk	2,260,000	Skimmed milk	12,000	Skimmed milk	1,450,000
	Milk 3·8 %	2,280,000	Milk 4·1 %	14,100	Milk 4·1 %	1,580,000
	Cream 10 %	2,620,000	Cream 12 %	13,200	Cream 12 %	1,390,000
			Cream 22 %	14,300	Cream 22 %	1,830,000

Temp. of 10 min. exposures ° C.			Temp. of 10 min. exposures ° C.			Temp. of 10 min. exposures ° C.			Time of holding at 60° C. min.
55	Skimmed milk	+ + +	65	Skimmed milk	+ +	66	Skimmed milk	+ +	5
	Milk	+ + +		Milk	+ +		Milk	+ +	
	Cream	+ + +		Cream 12 %	+ +		Cream 12 %	+ +	
				Cream 22 %	+ +		Cream 22 %	+ +	
58	Skimmed milk	+ + +	67	Skimmed milk	+ +	69	Skimmed milk	+ +	10
	Milk	+ + +		Milk	+ +		Milk	+ +	
	Cream	+ +		Cream 12 %	+ +		Cream 12 %	+ +	
				Cream 22 %	+ +		Cream 22 %	+ +	
60	Skimmed milk	+ + +	69	Skimmed milk	+ +	71	Skimmed milk	+ −	15
	Milk	+ + +		Milk	+ +		Milk	− −	
	Cream	+ + +		Cream 12 %	+ +		Cream 12 %	+ −	
				Cream 22 %	+ +		Cream 22 %	+ −	
62	Skimmed milk	+ + +	71	Skimmed milk	− −	73	Skimmed milk	− −	20
	Milk	+ + +		Milk	− −		Milk	− −	
	Cream	+ + −		Cream 12 %	− −		Cream 12 %	− −	
				Cream 22 %	+ +		Cream 22 %	− −	
64	Skimmed milk	+ − −	73	Skimmed milk	−	75	Skimmed milk	− −	25
	Milk	+ − −		Milk	− −		Milk	− −	
	Cream	+ + −		Cream 12 %	− −		Cream 12 %	− −	
				Cream 22 %	− −		Cream 22 %	− −	
66	Skimmed milk	+ − −	75	Skimmed milk	− −	77	Skimmed milk	− −	30
	Milk	+ + −		Milk	− −		Milk	− −	
	Cream	− − −		Cream 12 %	− −		Cream 12 %	− −	
				Cream 22 %	− −		Cream 22 %		
67½	Skimmed milk	− − −	77	Skimmed milk	− −	79	Skimmed milk	− −	
	Milk	− − −		Milk	− −		Milk	− −	
	Cream	− − −		Cream 12 %	− −		Cream 12 %	− −	
				Cream 22 %	− −		Cream 22 %	− −	
						81	Skimmed milk	− −	
							Milk	− −	
							Cream 12 %	− −	
							Cream 22 %	− −	

(B) Fixed temperature, variable time

Substrate	Culture No. 15	Culture No. 17	Culture No. 31
Skimmed milk	49,000	51,000	3,560,000
Milk 3·8 %	45,000	62,000	3,400,000
Cream 10 %	48,000	42,000	4,300,000

Substrate			
Skimmed milk	− − + +	+ + + +	+ + + +
Milk	+ + + +	+ + + −	+ + + +
Cream	+ + + +	+ + +	+ + + +
Skimmed milk	− − − −	+ + −	+ + + +
Milk	.	+ + − −	+ + + −
Cream	− − − −	+ − − −	+ + − −
Skimmed milk	− − − −	+ − − −	+ + + +
Milk	+ − − −	− − − −	+ + −
Cream	− − − −	− − − −	+ − − −
Skimmed milk	− − − −	− − − −	+ + − −
Milk	− − − −	− − − −	+ + + −
Cream	− − − −	− − − −	− − − −
Skimmed milk	.	.	+ + − −
Milk	.	.	+ + + +
Cream	.	.	− − − −
Skimmed milk	.	.	− − − −
Milk	.	.	− − − −
Cream	.	.	− − − −

very unlikely that any observed differences in heat resistance could be due to variations in the number of organisms initially present. The results of both increasing the temperature of holding for a fixed time (A) and increasing the time of holding at a fixed temperature (B) were somewhat inconsistent. Nevertheless, it is clear that the increase in the butterfat content did not increase the resistance of the micro-organisms to heat to any significant extent; in fact, the tendency in some instances was for the heat resistance to be slightly less in cream than in either the whole or the skimmed milk.

SUMMARY

1. A review of the literature shows that the destruction of living micro-organisms by heat is a complex process, and that the problem is particularly difficult when it is applied to the heat resistance of spores.

2. The problems involved in the method of heating and in the choice of a container for the inoculated substrate for heat-resistance tests are discussed, with special reference to the difficulties encountered at high temperatures such as are used in studies with spores of aerobic bacteria. Four different types of containers, namely, (*a*) glass capillary tubes, (*b*) glass ampoules or bulbs, (*c*) glass Pasteur pipettes and (*d*) corked test tubes, have been tested, and the latter were found to fulfil the necessary requirements.

3. A trial test of the heat resistance was conducted on nearly forty strains of aerobic sporeformers isolated from canned milk products, in order to obtain general information regarding the difficulties which might be expected in later and more detailed studies. Many of the strains were found to be exceedingly heat resistant. No dormancy was apparent when the spores were heated and subsequently incubated in milk products. "Skips" were only observed to any great extent among strains of *B. licheniformis*.

4. Special studies of the heat resistance of three strains of *B. subtilis* which had produced bitterness and thinning in canned cream were made. Details are given of the methods of preparing spore suspensions for these tests and of preparing the substrates for subsequent inoculation in such a way that equal concentrations of spores are present in the final subsamples.

5. The effect of the reaction of the substrate on the destruction of two of these strains of *B. subtilis* by heat has been examined, but over the narrow range of *p*H tested the results, which have been expressed as survival percentages, lack consistency.

6. The results of similar experiments designed to study the effect of increasing butterfat percentage on the heat tolerance of *B. subtilis* also failed to show uniformity. A second method of testing the effect of this variable, which involved the use of plate counts, was explored. The plate count tests showed more definitely than the survival percentage, that an increase in the butterfat content of the substrate did not increase the resistance of the spores to heat.

7. The outstanding findings of these studies has been the extremely high heat resistance of the aerobic sporeformers used in the investigation. It is perhaps not surprising, therefore, that the small changes in the *p*H and butterfat content of the substrates failed to show clear-cut differences in resistance.

8. In parallel studies it was, however, found that an increase in the butterfat content of the substrate failed even to increase the heat resistance of certain non-sporeforming organisms, which had relatively lower thermal death-points.

The author wishes to acknowledge the encouragement and valuable criticism from Dr N. C. Wright. She also wishes to thank Dr T. Gibson for his help during the course of this work.

REFERENCES

(1) BIGELOW, W. B. & ESTY, J. R. (1920). *J. infect. Dis.* **27**, 602.
(2) ESTY, J. R. & MEYER, K. F. (1922). *J. infect. Dis.* **31**, 650.
(3) BURKE, G. S. (1923). *J. infect. Dis.* **33**, 274.
(4) ESTY, J. R. & WILLIAMS, C. C. (1924). *J. infect. Dis.* **34**, 516.
(5) DICKSON, E. C., BURKE, G. S., BECK, D. & JOHNSTON, J. (1925). *J. infect. Dis.* **36**, 472.
(6) WILLIAMS, O. B. (1929). *J. infect. Dis.* **44**, 421.
(7) SOMMER, E. W. (1930). *J. infect. Dis.* **46**, 85.
(8) MAGOON, C. A. (1926). *J. Bact.* **11**, 253.
(9) ——(1926). *J. infect. Dis.* **38**, 429.
(10) WILLIAMS, F. T. (1932). *J. Bact.* **32**, 589.
(11) MORRISON, E. W. & RETTGER, L. F. (1930). *J. Bact.* **20**, 299.
(12) MORRISON, E. W. & RETTGER, L. F. (1930). *J. Bact.* **20**, 313.
(13) CURRAN, H. R. (1935). *J. infect. Dis.* **56**, 196.
(14) HOWAT, G. R. & WRIGHT, N. C. (1934). *Biochem. J.* **28**, 1336.
(15) NICHOLS, A. A., HOWAT, G. R. & JACKSON, C. J. (1937). *J. Dairy Res.* **8**, 331.
(16) MATTICK, A. T. R. & NICHOLS, A. A. (1935). *J. Dairy Res.* **6**, 125.
(17) BROWN, C. W. & PEISER, K. (1916). *Tech. Bull. Mich. agric. Exp. Sta.* no. 30.

(*MS. received for publication* 20 *June* 1940)

637.127.6

265. ON THE USE OF VARIOUS SERA FOR THE DETERMINATION OF THE SOLUBLE CALCIUM AND PHOSPHORUS OF MILK

By G. T. PYNE
Dairy Chemistry Department, University College, Cork

The soluble constituents of milk, and in particular phosphorus and calcium, are still commonly determined by the analysis of one of a variety of milk sera such as milk dialysate, rennet whey, papain whey and the mercuric chloride borax serum. Little or no information is available as to the relative trustworthiness of the values obtained; certain observations in the literature indicate, however, that in fact they diverge. The results of György (1) on the phosphorus content of whey and those of Michaelis & Rona (8) on its calcium content indicate that both are low compared with the corresponding values for milk ultrafiltrate, and the writer (11) has more recently found similarly low values for the phosphate of whey compared with that of milk dialysate. While no direct comparisons seem to have been made between papain serum and others, the use of this serum by Kometiani (2) to investigate the effect of heating of milk on its soluble constituents has given results so much at variance with those generally accepted as to throw doubt on its suitability, at least for this purpose.

A comparative study of the various methods appeared to be needed. The present paper records the results of such an examination.

Sera examined

These were milk dialysate, in the static form employed by Lampitt & Bushill (4) (which was taken as the standard), rennet whey, papain whey, and the mercuric chloride borax serum of Lang & Miethke (5). The first was prepared by dialysis for 2 days at 0–5° C. of 20 ml. water, contained in a Cellophane sac, against 1 l. holder-pasteurized skim milk (pasteurized to improve keeping quality and to obviate subsequent possible liberation of phosphate from esters through phosphatase action during preparation of other sera). To avoid the necessity for dilution corrections the other three sera were afterwards prepared from the same previously dialysed milk. Rennet whey was prepared at 30–35° C. by addition of 0·5 % dialysed commercial rennet solution, and the papain serum at 50° C. after addition of 5 % of an approx. 7·5 % solution of B.D.H. papain purified according to the instructions of Kometiani (2). Stoppered vessels were employed in each case, and as soon as a firm curd was obtained the material was cooled, shaken, and filtered or

centrifuged clear according to the quantity available. The first few ml. of filtrate were rejected. The Lang & Miethke serum, used to determine soluble phosphate only, was prepared by adding to 5 ml. milk in a 50 ml. graduated flask, a few ml. of water, followed by 10 ml. $HgCl_2$ reagent (150 ml. saturated $HgCl_2$, 70 gr. cryst. sodium acetate and 10 gr. NaCl) and 15 ml. 4 % borax, diluting to mark, mixing, allowing to stand a short time, and filtering.

As the phosphate content of whey at various degrees of acidity has been taken (cf. Ling(7)) to be a measure of that of the corresponding milk, some observations have also been included on the relative values of the phosphate and calcium contents of the various sera from acidified milks. The latter were prepared by running in with constant mechanical stirring some 17 and 34 ml. $N/1$ lactic acid respectively into further 1 l. samples of the same pasteurized skim milk employed for the preparation of the normal sera. All samples, both fresh and acidified, were subsequently dialysed simultaneously under identical conditions, and treated later in similar fashion for the preparation of the other sera. Results for the sera from the acidified milks were of course corrected for dilution resulting from the added acid.

Analytical methods

These were essentially the micro-methods used by Lang & Miethke(5) and Miethke & Levecke(9) for inorganic phosphate and calcium of milk, based on the application of the Fiske and Subbarow and the Cramer and Tisdall methods to trichloracetic acid filtrates.

Certain additional precautions were taken to ensure greater accuracy and in some instances additional modification was found to be necessary. The removal of supernatant liquid from the centrifuge tubes was carried out very slowly using fine glass siphons provided with turned-up tips, and collecting in a reception vessel whose level was maintained about 2–3 mm. below that of the siphon tip. The progressive slowing up of siphoning as the narrow tip of the centrifuge tube was approached eliminated the danger of disturbing any sediment and rendered supervision of the siphoning unnecessary.

Phosphate must be determined fairly rapidly after preparation of the trichloracetic filtrates in order to avoid high results arising from hydrolysis of phosphatic esters. With whey and papain sera the direct colorimetric determination of phosphate in trichloracetic filtrates had to be modified, as these filtrates usually contained sufficient nitrogenous matter to give turbid solutions with the molybdate reagent. An intermediate separation of the phosphate by adding magnesia mixture to the trichloracetic filtrate, standing overnight, centrifuging, decanting and rejecting the supernatant liquid was therefore practised in such cases.

The accuracy of these methods was checked on known mixtures and is considered to be within 2 %. Estimations were made at least in duplicate. Results are summarized in Table 1.

Table 1

		Soluble constituents %											
		A		*B*		*C*		*D*		*E*		Average	
		Inorg.		Inorg.		Inorg.		Inorg.		Inorg.		Inorg.	
Serum	*p*H range	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca
Dialysate	6·56–6·65	57	32	60	39	57	34	65	35	56	35	59·0	35·0
Rennet whey		53	30	60	38	53	34	61	32	53	36	56·0	34·0
Papain whey		50	31	58	35	54	35	59	32	47	30	53·6	32·6
$HgCl_2$-borax		65	—	65	—	—	—	—	—	64	—	(64·7)	—
Dialysate	5·89–6·00	78	54	79	63	78	58	82	59	—	—	79·2	58·5
Rennet whey		75	53	77	67	76	61	79	61	—	—	76·8	60·5
Papain whey		72	56	75	58	75	63	75	53	—	—	74·2	57·5
$HgCl_2$-borax		78	—	78	—	—	—	—	—	—	—	(78)	—
Dialysate	5·28–5·60	97	74	91	83	99	85	100	94	—	—	96·8	84·0
Rennet whey		96	77	93	89	97	89	99	98	—	—	96·3	88·3
Papain whey		93	81	89	80	99	96	100	92	—	—	95·3	87·2
$HgCl_2$-borax		95	—	85	—	—	—	—	—	—	—	(90)	—
Total inorg. P and total Ca of milks as mg./100 ml.		74	151	74	123	69	130	75	112	67	139	71·8	131

Discussion of sera results

Taking the values given by dialysis as the standard for comparison, rennet whey appears to be the most satisfactory of the sera. For fresh milk it yields results which are on the whole a little low for phosphate but almost correct for calcium. Papain serum is still lower in phosphate and is also slightly low in calcium, while the mercuric chloride borax serum is distinctly high in phosphate.

With increasing acidity of the milk the relative positions of the various sera alter. The values for phosphate in dialysate, whey and papain serum tend to coincide, and those for the mercuric chloride serum also show closer agreement. With calcium values acidity appears to exercise a similar but more marked effect, the whey and papain serum contents of this element differing little on the average from that of the dialysate at moderate degrees of acidity, but exceeding it at the higher range. The papain values are throughout somewhat lower, and decidedly more irregular than those for whey, particularly with the acidified milks.

The high values given by the mercuric chloride borax serum with unacidified milk are no doubt due to a disturbance in the phosphate equilibrium of milk, accompanied by solution of some of the previously insoluble phosphate, following the addition of the deproteinizing reagents. Apart from such specific interactions, dilution itself may also play a part. Results show closer agreement for the acidified milks, as might be expected, where both the proportion of insoluble phosphate is smaller, and the effect of the added reagents, particularly borax, is likely to have the reverse effect of precipitating some of the soluble phosphate by making the reaction of the whole more alkaline. Such a net reduction in soluble phosphate is in fact observable at the higher acidity.

ACTION OF PAPAIN ON BOILED MILK

The consistently low results which had been obtained for the soluble phosphate and calcium of fresh milk by the use of papain made it of interest to examine also the effect of this enzyme on heated milk, in the hope of arriving at an explanation of the anomalous results obtained by Kometiani (2). Using papain serum to determine the soluble phosphate and calcium of milk, this author found that heating (even boiling for half an hour) brought about no reduction in the concentration of those constituents. Preliminary experiments confirmed this unexpected result: the soluble calcium and phosphate content of boiled milk determined by the use of papain was frequently found to be even higher than before boiling. Results of more extensive experiments, which include some data on the effect of the time of action of the enzyme on raw and heated milks, are given in Table 2.

Table 2

Soluble constituents mg./100 ml.

	I		II						III			
	Inorg. P	Ca	Inorg. P			Ca			Inorg. P		Ca	
Time of enzyme action	—	—	10′	20′	80′	10′	20′	80′	5′	60′	5′	60′
Papain serum, raw milk	25·3	43·6	33·4	33·4	33·1	53·0	54·2	52·4	29·0	—	47·3	—
Papain serum, milk 100° C. × 30′	49·0	99·0	39·9	44·8	46·6	71·4	83·0	87·1	29·5	40·2	48·3	75·6

From these results it appears that, while papain yields with raw milk a serum whose phosphate and calcium content is more or less independent of the duration of the enzyme action, with boiled milk it yields a serum containing increasing amounts of these constituents with increasing time of contact of curd and serum. With such milk even quite a short time of reaction, little more than sufficient to form a stiff curd, is capable of producing a marked increase in soluble phosphate and calcium. Kometiani's results may perhaps be explainable therefore as a case in which the actual fall in soluble phosphate and calcium due to boiling was roughly balanced by the rise in these constituents brought about by the peculiar solvent action of papain under the conditions of enzyme strength and time of contact employed.

The source of the extra phosphate and calcium appears to be largely the insoluble or colloidal phosphate of the boiled milk, since no similar differences (or only very slight ones of 1–2 mg. P/100 ml.) were obtained between the phosphate contents of the papain serum from boiled and unboiled solutions of (*a*) 3 % calcium caseinate (from Hammarsten casein) and (*b*) milk which had been oxalated and dialysed to free it from phosphates. That no great part of the phosphate could arise from the degradation of the heated casein was fairly evident from consideration of the amount of extra phosphate brought into solution, which in some cases was found to exceed the total organic phosphorus of the casein.

There remained a slight doubt, however, as to whether some at least of the extra phosphate might not owe its existence to the hydrolysis of phosphorus-containing degradation products of casein by the strongly alkaline magnesia mixture used for the intermediate separation of the phosphate. An alternative direct method of analysis was therefore sought which would obviate the intermediate use of magnesia mixture. Of many protein precipitants examined the most satisfactory was found to be the picric acid reagent used by Lens-strup (6). This was capable of removing most, but not quite all, of the nitrogenous matter which made the direct estimation of phosphate with molybdate impracticable. Using picric acid filtrate and the more sensitive Kuttner & Lichenstein (3) colorimetric molybdate technique in order to reduce the quantity of serum required to a minimum, it was found possible to obtain direct readings for the phosphate in the papain serum of boiled milk, provided the tints were compared in the colorimeter as soon as possible after adding the molybdate and stannous chloride reagents. The standard for comparison was adjusted to about the same phosphate concentration and the same concentration of picric acid as the solution to be tested. The results agreed, within the limits of experimental error, with those obtained by the magnesia mixture technique. It is clear, therefore, that the high values for phosphate obtained from the papain sera of boiled milk represent inorganic phosphate actually present in these sera at the moment of filtering.

It may therefore be concluded that the additional phosphate in the papain serum of boiled milk is derived almost entirely from the solution of colloidal calcium phosphate either released from the boiled calcium caseinate-phosphate complex by the action of papain or perhaps, as argued by Kometiani (2) in another connexion, partly or wholly released from this complex by the previous boiling and subsequently brought into solution by the presence of soluble protein degradation products split off by papain.

Whether all the extra calcium arises from the same source is not certain. The ratio Ca/P for the additional calcium and phosphate brought into solution in this way is about 1·8 instead of the value 1·5 required for tricalcium phosphate, so that the possibility that a fraction arises from the presence in solution of calcium-containing degradation products of casein cannot be excluded.

It is of interest to recall that György (1) has noted a somewhat similar but slower increase in soluble phosphate and calcium as a result of the action of pancreatic ferment on calcium caseinate-phosphate complex, and has shown that this increase also takes place at the expense of the inorganic colloid. György drew the conclusion that this behaviour suggested evidence of a chemical rather that a merely colloidal union between the calcium phosphate and the calcium caseinate of milk. The present writer (10) has already offered additional evidence in favour of this view, and intends to deal in a later paper with the bearing of the present observations on this question.

SUMMARY

A comparison of the value of various milk sera for the determination of the soluble phosphate and calcium of milk, using dialysis at 0–5° C. as a standard method, gave the following results:

(i) Rennet whey was the most satisfactory; with fresh milk it yields fairly accurate values for calcium, slightly low (average 5 %) for phosphate. With increasing acidity of the milk both sets of values tend to rise somewhat relatively to those of the dialysate, phosphate approaching the true value and calcium rising slightly above it.

(ii) Mercuric chloride borax serum gives high results for the soluble phosphate of fresh milk. With increasing acidity of the milk, the results fall steadily.

(iii) Papain whey gives somewhat low results for both the phosphate and calcium of raw and pasteurized milk, with the same tendency as rennet whey to yield higher and improved results as the acidity of the milk increases. With boiled milk it gives abnormally high results for both constituents. The additional phosphate and calcium appear to arise from the solution of colloidal calcium phosphate.

REFERENCES

(1) GYÖRGY (1923). *Biochem. Z.* **142**, 1.
(2) KOMETIANI (1931). *Milchw. Forsch.* **12**, 433.
(3) KUTTNER & LICHTENSTEIN (1930). *J. biol. Chem.* **86**, 671.
(4) LAMPITT & BUSHILL (1933). *Biochem. J.* **27**, 711.
(5) LANG & MIETHKE (1932). *Milchw. Forsch.* **14**, 195.
(6) LENSTRUP (1926). *J. biol. Chem.* **70**, 193.
(7) LING (1936). *J. Dairy Res.* **7**, 145.
(8) MICHAELIS & RONA (1909). *Biochem. Z.* **21**, 114.
(9) MIETHKE & LEVECKE (1932). *Milchw. Forsch.* **13**, 535.
(10) PYNE (1934). *Biochem. J.* **28**, 940.
(11) —— (1935). *Sci. Proc. R. Dublin Soc.* N.S. **24**, 223.

(*MS. received for publication* 17 *April* 1940)

637.333:637.128

266. THE INFLUENCE OF "MASTITIS" UPON THE YIELD AND QUALITY OF CHEDDAR CHEESE[1]

By C. K. JOHNS,[2] T. J. HICKS,[3] and C. A. GIBSON[4]
Department of Agriculture, Ottawa, Canada

It is generally accepted that milk which is macroscopically abnormal is unsuitable for Cheddar cheese-making. It has also been reported (1,2) that latent infection with *Streptococcus agalactiae* renders the milk undesirable for this purpose, both yield and quality of cheese being adversely affected. On the other hand, it has been claimed (3) that cheese equal in yield and quality to that from normal milk may be obtained by the use of larger amounts of starter or by the addition of mineral acid to the milk.

While infection with *Str. agalactiae* is no doubt the commonest form of udder abnormality, recent studies (4,5,6,7,8) indicate that in some herds appreciable numbers of "Streptococcus-free" cows yield foremilk reacting positively to the "indirect" tests commonly used to diagnose chronic mastitis. This group includes cows suffering from chronic staphylococcus mastitis as well as those from which no recognized pathogen has been isolated. While by ordinary criteria such milks would be judged acceptable for market milk purposes, the fact that they frequently react abnormally to rennet (9) suggested that they might also be inferior to normal milk for cheese-making. The Animal Husbandry Division herd at the Central Experimental Farm offered an excellent opportunity for studying this point, since detailed records of udder condition were available since 1935. On the basis of these records, supplemented by additional testing prior to and during the cheese-making periods, three groups, with six to ten cows in each, were established:

A. Normal—negative daily strip-cup record; no evidence of bacterial infection; catalase rarely over 30% by Hastings tube method (6).

B. Abnormal—strip-cup record negative or rarely mildly positive; frequent high values for catalase and *p*H; *Str. agalactiae* absent from repeated samplings.

C. Agalactiae—*Str. agalactiae* previously isolated from milk on at least two occasions; strip cup rarely positive; latent infection with no pathogens detected during the experimental period.

[1] Contribution from the Division of Bacteriology and Dairy Research, Science Service (No. 110, Journal Series); the Division of Animal Husbandry, Experimental Farms Service; and the Dairy Products Division, Marketing Service.

[2] Associate Bacteriologist, Division of Bacteriology and Dairy Research.

[3] Senior Dariry Produce Grader, Dairy Products Division.

[4] Creamery Manager, Division of Animal Husbandry.

EXPERIMENTAL

The cheese-making value of the milks was studied in two series of comparisons, the standard procedure for the manufacture of Canadian Cheddar cheese being followed throughout. As the plate count of the herd milk averaged around 3000 per ml., steps were taken to increase the bacterial content. In the first series, small amounts of starter were introduced into the shipping cans in which the milk was held overnight; in the second series, in order to obtain a more heterogeneous flora, shipping cans were washed but not sterilized. To encourage bacterial multiplication, in both series the cans of milk were held overnight at around 60° F.

Chemical analyses were conducted on the milk, whey, curd and cheese by the Chemistry Division, Science Service. The nitrogen distribution in the milk was determined by the methods of Rowland (10), and chlorides by the open Carius method (11). The *p*H values were determined by us with the glass electrode, and catalase by the Hastings (6) tube method, incubating at room temperature for 24 hours.

The weight of cheese off the press and after 14 days curing was recorded. The latter figures were used in calculating the yield of cheese, since chemical analyses were made at this time. The theoretical yield was determined by the formula (12)

$$\text{Yield} = (1{\cdot}4\ \text{fat} + 0{\cdot}9)\ N,$$

where N varies with moisture content of cheese from 1·53 for 33% to 1·68 for 39%. To facilitate comparison, the actual/theoretical yield for each class of milk was expressed as a percentage of that for the normal milk.

SERIES I (JANUARY 1939)

Influence on yield of cheese

A portion of the data obtained is shown in Table 1. It is evident that the yield from both "abnormal" and "agalactiae" milks is inferior to that from normal milk. Furthermore, with three of the four pairs of vats, the shrinkage during the first two weeks of curing was definitely less with the normal milks. The lowered yield appears to be closely correlated with the lower content of solids-not-fat and casein in the even-numbered vats. Vat 8 ("agalactiae") is the only one showing a "casein number" $\left(\frac{\text{casein } N}{\text{total } N} \times 100\right)$ below the limit of 78 set by Rowland (10).

Influence upon quality of cheese

With the exception of vat 8, all vats worked normally, and no appreciable differences in quality were noted between the cheese from the three groups (Table 2). Vat 8 worked slowly and an extra 0·6% of starter was added shortly before setting to hasten acid production. Although acid developed rapidly after setting, the curd failed to shrink properly and was "dead" and "soggy".

Table 1. *Data from Series I,* 16–26 *January* 1939

Type of milk ...	Normal				"Abnormal"			"Agalactiae"
Vat no. ...	1	3	5	7	2	4	6	8
% Starter	0·5	0·87	1·25	1·25	0·0	0·75	1·0	1·6
Acid at setting	0·22	0·21	0·21	0·21	0·23	0·21	0·21	0·21
Minutes to 1st coagulation	6	7	7	8	6	9	7	7
Cell count (thousands)	127	95	159	—	440	571	444	846
Catalase %	12	25	9	14	24	53	28	47
*p*H at start	—	—	6·65	6·72	—	—	6·65	6·79
MILK:								
Fat %	3·37	3·92	3·57	3·49	3·55	3·81	3·72	3·41
S.N.F. %	8·86	9·32	9·37	8·98	8·60	8·50	9·08	8·43
Casein %	2·70	2·78	2·82	2·71	2·62	2·67	2·70	2·61
Casein no.	80·1	80·1	79·5	79·8	79·0	79·2	79·0	76·1
Chlorides %	0·089	0·086	0·085	0·084	0·092	0·087	0·093	0·113
WHEY:								
Fat %	0·26	0·23	0·20	0·25	0·29	0·24	0·25	0·21
S.N.F. %	6·37	6·47	6·77	6·52	6·37	6·62	6·63	6·45
CHEESE:								
Moisture %	36·07	36·65	37·79	37·24	36·49	36·79	36·32	38·77
Yield per 100 lb. milk	9·94	10·78	10·28	10·12	9·56	10·30	10·04	9·62
Theoretical yield	9·17	10·35	9·73	9·46	9·49	10·13	9·84	9·50
Actual/theoretical %	108·40	104·15	105·65	106·96	100·74	101·68	102·03	101·26
Average %		106·29				101·48		101·26
% yield compared with normal milk		100·0				95·47		95·26

Table 2. *Flavour scores on cheese made* 16–26 *January* 1939

Type of milk ...	Normal				"Abnormal"			"Agalactiae"
Vat. no. ...	1	3	5	7	2	4	6	8
17 February 1939	39·0	40·2	39·0	40·0	40·0	39·0	39·2	38·2
24 April 1939	39·3	40·0	39·0	39·0	39·0	39·0	38·0	38·0
16 September 1939	38·8	39·0	38·7	39·0	38·9	39·5	38·9	36·0
20 January 1940	36·0	38·0	37·0	38·5	37·0	37·0	37·1	36·0

N.B. To be classed as first grade under the Dominion grading regulations, cheese must have a flavour score of not less than 39 points and a total score of not less than 92 points.

A considerable quantity of rich-looking whey drained off when in press. The texture of this cheese was criticized as being "open" and the body as being "slightly weak". The flavour score for this vat was lowest of the eight vats at the first grading, and dropped to 36 points after 8 months. However, vat 1, made from normal milk, also dropped to 36 points, at the final grading, hence the deterioration in flavour may not be attributable to the type of milk used.

SERIES II (MAY 1939)

In order to confirm the results obtained in series I, a second series of comparisons of the three different types of milk was made in May 1939. In addition, the effect of using one-third "abnormal" or "agalactiae" milk was also studied.

Influence on yield of cheese

Although there is some variation between replicate vats within the various groups (Table 3) here again the yield is definitely reduced when either "abnormal" or "agalactiae" milk is used. As in the first series, this appears to be

Table 3. *Data from Series II*, 1–13 *May* 1939

Type of milk ...	Normal				1/3 "Abnormal"		1/3 "Agalactiae"		"Abnormal"		"Agalactiae"	
Vat no. ...	1	4	7	10	2	8	5	11	3	9	6	12
% Starter	0·5	1·5	1·3	1·35	1·5	1·5	1·35	1·25	1·35	1·35	1·6	1·36
Acid at setting	0·22	0·23	0·22	0·22	0·23	0·22	0·23	0·22	0·23	0·22	0·23	0·22
Minutes to first coagulation	8	9	8·5	8·8	8·5	7·5	9	7	6	7	8	8
Cell count (thousands)	105	169	175	169	222	524	338	391	1227	540	794	888
Catalase %	9	26	9	14	22	44	23	23	34	39	30	31
*p*H at start	6·57	6·48	6·53	6·52	—	6·53	6·55	6·56	6·55	6·60	6·50	6·61
MILK:												
Fat %	3·75	3·9	3·8	3·8	4·25	4·2	4·2	4·1	3·6	3·6	3·8	3·5
S.N.F. %	9·04	9·11	9·09	9·11	9·15	8·92	9·12	9·00	8·85	8·75	—	8·78
Casein %	—	2·67	2·57	2·65	2·51	2·65	2·58	2·56	2·46	2·43	2·38	2·27
Casein no.	77·5	78·5	78·0	79·3	75·1	77·5	78·2	78·5	75·6	76·3	77·3	75·1
Chlorides %	0·078	0·078	0·081	0·083	0·078	0·084	0·080	0·083	0·097	0·095	0·092	0·090
WHEY:												
Fat %	0·23	0·25	0·20	0·25	0·25	0·24	0·28	0·27	0·22	0·22	0·26	0·28
S.N.F. %	6·79	6·65	6·55	6·75	6·72	6·61	6·69	6·61	6·43	6·46	6·44	6·49
CHEESE:												
Moisture %	34·75	34·37	35·41	36·99	35·19	34·62	34·99	37·07	36·55	36·71	33·81	36·15
Yield per 100 lb. milk	9·84	9·69	9·55	9·84	10·32	9·78	9·96	9·87	9·22	8·82	8·67	8·61
Theoretical yield	9·34	9·25	9·21	9·21	10·33	9·81	9·84	10·31	9·64	8·87	8·68	8·99
Actual/theoretical yield %	105·46	104·76	103·69	106·84	100·0	99·69	101·22	95·73	95·64	99·44	100·0	95·77
Average %	105·19				99·85		98·48		97·54		97·89	
% Yield compared with normal milk	100·0				94·91		93·61		92·72		93·05	

correlated with lower solids-not-fat and casein contents. Just why the addition of one-third "abnormal" or "agalactiae" milks should cause so great a reduction in yield is not apparent.

Influence on quality of cheese

In contrast to the findings in the first series, the cheese made from "agalactiae" milk were in no respect inferior in quality to those made from normal milk. Table 4 shows the flavour scores for the twelve vats.

Table 4. *Flavour scores on cheese made* 1–13 *May* 1939

Type of milk ...	Normal				1/3 "Abnormal"		1/3 "Agalactiae"		"Abnormal"		"Agalactiae"	
Vat no. ...	1	4	7	10	2	8	5	11	3	9	6	12
27 May 1939	39·5	39·3	38·0	39·0	39·0	38·0	38·9	39·0	39·0	38·5	39·1	39·0
16 September 1939	40·0	39·0	39·5	39·0	38·8	38·5	38·9	38·8	38·5	39·0	39·5	39·2
20 January 1940	39·0	38·0	38·0	38·3	38·5	38·0	39·0	37·9	38·3	38·1	38·5	38·4

Discussion

It should be emphasized that none of the milks used in these studies showed any macroscopic evidence of abnormality.[1] With the possible exception of vat 8 in series I, they appear to have been less abnormal in chemical composition than those employed by Mattick *et al.* (1) and Price (2). Nevertheless, the lowering of yield of cheese is comparable to that reported by these workers, although the adverse effect upon quality which they reported was less evident in the present studies.

The findings reported by Marquardt & Hucker (3) are very difficult to understand. The "agalactiae" milks they employed were distinctly more abnormal than those used in our studies. Although no data were presented, all available evidence would suggest that such milks were definitely inferior in solids-not-fat and casein. It is therefore surprising that a normal yield of cheese should be obtained from such milks. Whether or not the different amounts of starter added, and the higher moisture content of the mastitis cheese (averaging 37·96% v. 36·0% for the control vats) were taken into consideration in comparing yields is not indicated.

Abnormal milks of the types used in the present studies appear to be detected most readily by the catalase content or cell count. Unfortunately, our catalase tubes were not read until after 24 hr. at room temperature, and it is suspected that the unduly high values recorded in several instances for milks of low cell count are due to catalase production by bacteria. Subsequent studies have shown that with a shorter incubation period the catalase content checks more closely with the cell count and other evidence of abnormality. Studies are under way to determine the suitability of a simplified catalase test for factory use.

[1] In the case of vats containing "agalactiae" milk we were unable to isolate the streptococcus from any of the vat samples.

As a possible means of detecting milks of abnormal composition, the rennet coagulation test of Sommer & Matsen(13) was applied to samples of the vat milks (before the addition of the main body of starter) in the first series. While this test works well with samples from individual cows or quarters, with vat samples there were wide variations within the groups and considerable overlapping of values between groups. In the second series, the brom-cresol-purple-rennet test of Davis & McClemont(14) was tried out, but it also proved to be of limited value for this purpose.

The correlation between solids-not-fat content and yield of cheese has already been referred to. Further data furnished by one of us (C. A. G.) on the composition of samples taken on one day each month from the individual cows in the three groups have been analysed. These were taken between 22 September 1938 and 29 July 1939, the solids-not-fat content being determined from the Babcock test and lactometer reading by the formula,

$$\text{S.N.F.} = \frac{\text{Fat}}{5} + \frac{\text{Lactometer reading}}{4}.$$

In graphing these data, the curve for the average relationship of solids-not-fat to fat (based upon the values obtained by Kelly & Clement(15) from 250,000 analyses) was first drawn, then the number of samples in each group falling above or below the curve was determined. The results were as follows:

	Total no.	% above average	% below average
Normal milk	115	57·4	42·6
"Abnormal"	89	52·8	47·2
"Agalactiae"	57	43·9	56·1

These figures indicate a definite lowering of the solids-not-fat content in the "abnormal" milks and an even more marked lowering in the "agalactiae" milks.

Dahlberg *et al.*(16) have reported that "the percentage of casein was not decreased by latent mastitis". On the other hand, the comprehensive studies of Rowland(10,17) have shown a definite lowering of casein and of solids-not-fat content as a result of subclinical "agalactiae" mastitis. Our data also show reduction in casein in both "agalactiae" and "abnormal" milks, especially in the second series. Calculations of Rowland's "casein number" from Dahlberg's data clearly illustrate the divergent results obtained with different analytical methods. Rowland reports casein numbers from 77·3 to 80·7 (average 78·7) for individual cow samples of *normal* milk, while Dahlberg's data yield values as high as 88·5 for the average of samples *from a highly infected herd.*

Price(2) found a much higher fat content in the whey from "agalactiae" milk than from normal (0·6 v. 0·24%), and remarked, "Evidently the abnormally large manufacturing losses in the whey from the abnormal milk caused a marked decrease in the yield of the cheese per hundred pounds of milk". In our studies the smaller yield appears to be attributable to the lowered solids-not-fat content of the milk.

SUMMARY

Milk of normal appearance from cows having a latent infection with *Str. agalactiae* yielded definitely less cheese than that from normal cows. Milk from cows free from "agalactiae" infection, yet classed as "abnormal", behaved similarly. The lowered yield appeared to result from the lower casein and solids-not-fat contents of these "mastitis" milks.

With a single exception, the cheese made from these "mastitis" milks were not inferior in quality to those made from normal milks.

We are indebted to Mr R. K. Howson, B.S.A., Division of Bacteriology and Dairy Research, for technical assistance; to Mr Jos. Burgess, Chief, Grading and Inspection Service, Dairy Products Division, and his staff for scoring the cheese periodically; to the Animal Husbandry Division for furnishing the milk and facilities for manufacturing and ripening, and to the Chemistry Division, Science Service, for chemical analyses of the milk, whey and cheese.

REFERENCES

(1) MATTICK, A. T. R. *et al.* (1937). *Int. Dairy Congr. Berlin*, **11**, 283.
(2) PRICE, W. V. (1934). *Nat. Butter and Cheese J.* **25**, (21), 24.
(3) MARQUARDT, J. C. & HUCKER, G. J. (1937). *Tech. Bull. N.Y. St. agric. Exp. Sta.* no. 242.
(4) HASTINGS, E. G. & BEACH, B. A. (1937). *J. agric. Res.* **54**, 199.
(5) —— —— (1939). *J. agric. Res.* **58**, 543.
(6) JOHNS, C. K. & HASTINGS, E. G. (1938). *Canad. J. Res.* D, **16**, 6.
(7) MILLER, W. T. (1936). *N. Amer. Vet.* **17**, (3), 32.
(8) PETERSON, E. H. *et al.* (1938). *Cornell Vet.* **28**, 307.
(9) JOHNS, C. K. (1939). Unpublished data.
(10) ROWLAND, S. J. (1938). *J. Dairy Res.* **9**, 42.
(11) DAVIES, W. L. (1932). *Analyst*, **57**, 79.
(12) VAN SLYKE, L. L. & PRICE, W. V. (1932). *Cheese*, 2nd ed. New York: Orange Judd Publishing Co.
(13) SOMMER, H. H. & MATSEN, H. (1935). *J. Dairy Sci.* **18**, 741.
(14) DAVIS, J. G. & MCCLEMONT, J. (1939). *J. Dairy Res.* **10**, 94.
(15) KELLY, E. & CLEMENT, C. E. (1923). *Market Milk.* New York: John Wiley and Sons, Inc.
(16) DAHLBERG, A. C. *et al.* (1936). *Tech. Bull. N.Y. St. agric. Exp. Sta.* no. 239.
(17) ROWLAND, S. J. & ZEIN-EL-DINE, M. (1938). *J. Dairy Res.* **9**, 174.
(18) MCDOWALL, F. H. *et al.* (1937). *J. Dairy Res.* **8**, 31.
(19) HOOD, E. G. & WHITE, A. H. (1927). *Bull. Dom. Canad. Dep. Agric.* **79.**

(*MS. received for publication* 9 *May* 1940)

637.325

267. STUDIES ON THE CHEMISTRY OF CHEDDAR CHEESEMAKING

VII. THE MEASUREMENT OF THE ACIDITY OF CHEESE AND THE RELATION OF ACIDITY TO GRADING SCORE

By R. M. DOLBY, F. H. McDOWALL and W. RIDDET
Dairy Research Institute (N.Z.), Palmerston North, New Zealand

(With 5 Figures)

The acidity characteristics of Cheddar cheese, as described by cheese graders, range from "acid" through "normal" to "sweet". These descriptions are based on a qualitative examination of a sample of the cheese, and may be misleading in that they do not always distinguish between the effects of acidity, and of other factors, on the cheese, e.g. moisture and salt content of the cheese. An actual measurement of acidity in cheese may be made either by determination of hydrogen-ion concentration or by estimation of titratable acidity. While little work has been done in relating titratable acidity to quality of cheese, certain studies have shown(1,2) that there is a general relationship between *p*H of young cheese and the quality of the mature cheese. A comparison of the results of both hydrogen-ion concentration and titratable acidity measurements with grading observations on cheese should be of value in indicating the usefulness and limitations of the various methods. The present paper presents the results of such a comparison made on some 420 cheese comprising a complete season's make at the Dairy Research Institute experimental factory. Advantage has also been taken of the opportunity to compare two different methods of determining the titratable acidity of cheese.

Experimental

Time of making observations. Since the titratable acidity and *p*H of cheese change considerably during their life, the time at which determinations are carried out is of importance. It has been shown in an earlier paper in this series(3) that during the first few days after manufacture there is a rapid fermentation of the lactose in cheese, and a correspondingly rapid fall in *p*H. In most cases the whole of the lactose is fermented within 7–10 days, and there is no further acid production thereafter. It seemed preferable to make the various acidity determinations when a stable state had been reached than at an earlier stage when the *p*H was still falling. This point appears to have been overlooked by previous workers(1,2) who made estimations at from 3 to 10 days. In the present investigation 14 days was selected as the most suitable age for the first examination. Grading observations and determinations of both

titratable acidity and *p*H were made at this time and were repeated when the cheese was 3 months old.

Methods

Grading observations were made by members of the staff of the Institute factory.

Hydrogen-ion concentration (expressed as *p*H values) was determined with the quinhydrone electrode. The method used corresponded to the British Standard method (4) except that 1 ml. of water was added to 5 g. of cheese in forming the mush.

The titratable acidity of cheese was determined by two methods:

(1) The acidity of the water soluble extract (referred to later as "soluble acidity") was estimated by a modification of the method of Van Slyke & Bosworth (5) as follows:

12·5 g. of cheese were triturated with water at 50° C. and the mixture was transferred to a 250 ml. measuring flask, sufficient water being added to bring the liquid to the base of the neck. After shaking two or three times during 10 min., the flask was transferred to an incubator at 37° C. and after 15 min. the contents were made up to the mark. After a further 15 min. the flask was removed from the incubator, the fat was drawn off by suction and the cheese suspension was filtered through a fluted filter paper (Whatman No. 1). 50 ml. of the filtrate was titrated with *N*/10 NaOH using 1 ml. of 1 % phenolphthalein as indicator. The results are expressed as ml. *N*/10 NaOH per 100 g. of cheese.

(2) The total titratable acidity of the cheese ("total acidity") was estimated by the British Standard method (4) in which 2 g. of cheese are titrated while being ground in a mortar. The results are expressed as ml. *N*/10 NaOH per 100 g.

The second method was found to give a much less definite end-point than the first. Further, in the second method the colour of the cheese had a pronounced effect. Cheese coloured with annatto gave a higher titration than white cheese made under similar conditions. The magnitude of this effect is illustrated later in this paper (Fig. 5). The use of a colour standard does not appear practicable. Comparisons must therefore be limited to cheese containing the same amount of colouring matter. This constitutes a serious disadvantage of the method. Of the cheese included in the present investigation only twenty were white and the remainder were coloured by the addition of a standard quantity of annatto to the milk before renneting. Results for white and coloured cheese have been dealt with separately.

Results

The results have been set out diagrammatically in Figs. 1–3, which show the relationship between grading score at 3 months and the *p*H, soluble acidity and total acidity respectively at 14 days. Comparisons with 14-day gradings gave somewhat similar results, but have not been reproduced here as the 14-day grading must be considered mainly a forecast of the quality of

the cheese at maturity. In each figure the average grading scores of cheese falling within each zone of *p*H or acidity, are shown as points connected by dotted lines. A smooth curve drawn by grouping the mean values indicates the general trend of the relationship. The numbers of cheese falling in each zone of *p*H or acidity are also shown in histograms, where appropriate shading indicates the numbers in each group graded as "finest" (93 points and over), "first grade" (over 90 points and under 93 points) and "second grade" (90 points and under).

The comparison of grading score with *p*H value (Fig. 1) shows a definite falling off in average grading score with cheese of high *p*H values and a less sharp decline at the lower *p*H values. The maximum occurs at about *p*H 4·90. Results obtained on another season's make of cheese showed a similar type of

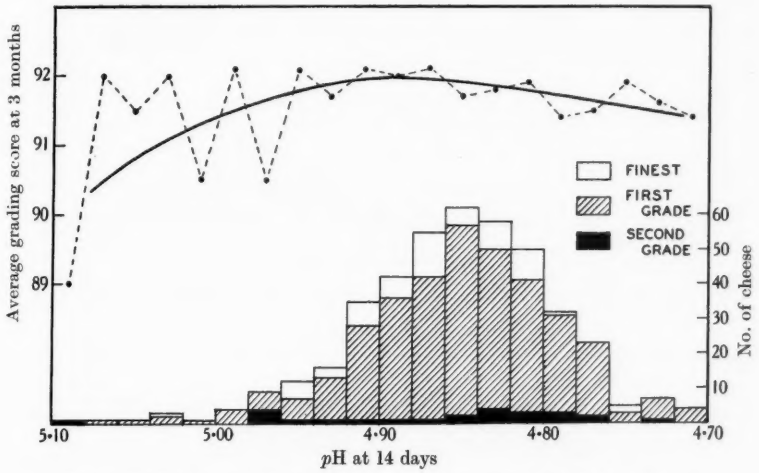

Fig. 1. Grading scores at 3 months of cheese of various *p*H values at 14 days.

curve with a rather more pronounced maximum at about *p*H 4·92–4·94. Points for individual cheese were found to spread widely on either side of the smoothed curve, a fact which is also indicated in the histogram. This is to be expected since acidity is only one of a number of factors controlling cheese quality.

The two methods of acidity determination gave similar results (Figs. 2, 3). With cheese of low acidity there was a rapid fall in average grading score as acidity decreased. There was, however, very little decrease in grading score for cheese of high acidity. An explanation of this is found when a direct comparison is made between acidity and *p*H (Figs. 4, 5). In these figures the mean acidities for cheese in each zone of *p*H values are plotted. Numbers of cheese are not shown as they are approximately the same as in Fig. 1. It will be seen that as the *p*H rose above 4·9 there was a rapid fall in acidity,

but with a decrease in *p*H value below 4·9 there was very little corresponding increase in acidity.

It would appear, therefore, that titratable acidity methods fail to distinguish over-acid from normal cheese. This view is supported by the observation that cheese of high titratable acidity did not show acid defects to any greater extent than those of normal titratable acidity.

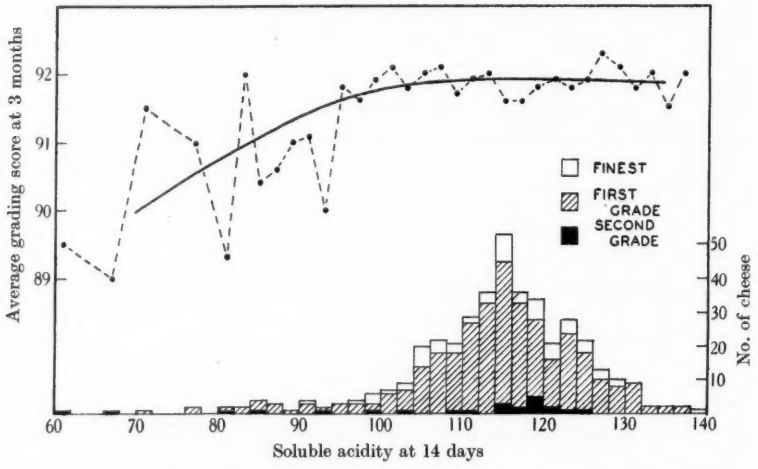

Fig. 2. Grading scores at 3 months of cheese of various acidities at 14 days.

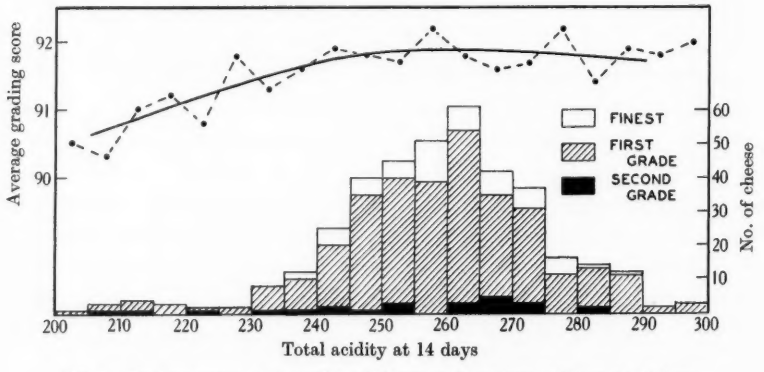

Fig. 3. Grading scores at 3 months of cheese with various acidities at 14 days.

The effect of colour of cheese on titratable acidity determined by the British Standard method is also illustrated in Fig. 5, which shows that white cheese give a much lower acidity reading than do coloured cheese of the same *p*H value. The number of results available for white cheese was not adequate to warrant the construction of a smooth curve.

It seemed possible that in comparing acidities of cheese with widely differing moisture and fat contents, a sounder basis of expressing the results would be as ml. $N/10$ NaOH per 100 g. fat-free dry matter instead of per

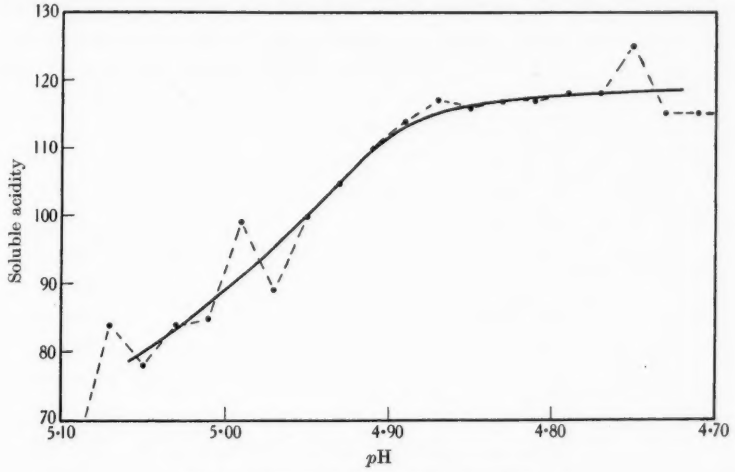

Fig. 4. Acidities of cheese of various pH values.

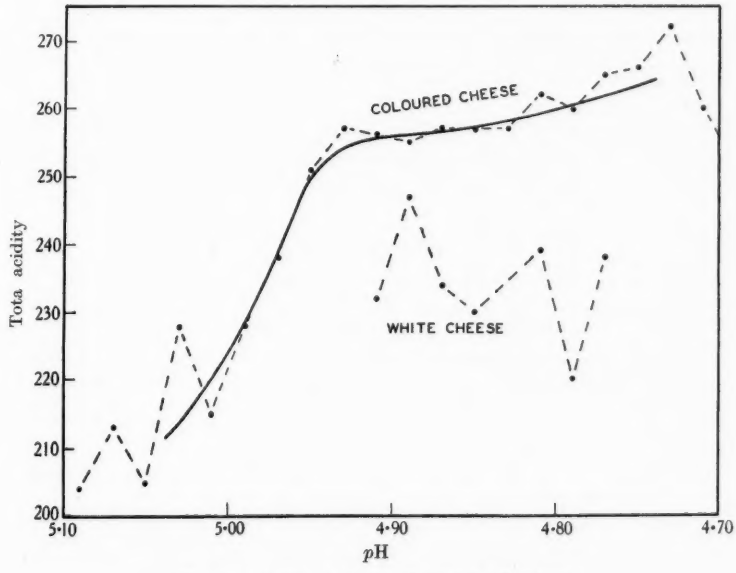

Fig. 5. Acidities of cheese of various pH values.

100 g. cheese. All the acidity results by both methods, re-calculated on this basis, were compared with the corresponding pH values. It was found that, while the relative values for individual cheese were to some extent altered,

the general shape of the curves and the spread of points from the average line was not appreciably affected.

The results of determinations of *p*H and titratable acidity on cheese 3 months old have not been reproduced here, as their relationship to cheese quality was found to be much less clearly defined than with the 14-day determinations. As the cheese matured, there was in general an increase in both *p*H and titratable acidity by both methods, but the increase varied considerably from one cheese to another.

Summary and conclusions

The *p*H of cheese at 14 days old appears to be the most useful means of measuring the extent of acid development in the cheese, and shows a very fair agreement with the observations of graders on mature cheese. Cheese with a *p*H value close to 4·90 at 14 days gained the highest average score at maturity.

Measurements of titratable acidity either on the aqueous extract of the cheese or directly on a paste of the cheese, readily distinguish cheese which are insufficiently acid, but do not clearly distinguish over-acid cheese or cheese of low *p*H value. The direct method of acidity titration has the disadvantage that the results are affected to such an extent by the colour of the cheese as to make impossible the comparison of results on cheese of different colour intensities.

*p*H or titratable acidity values for mature cheese are less useful indicators of the quality of the mature cheese than the values at 14 days.

The thanks of the authors are due to Mr A. K. R. McDowell who assisted in the analytical determinations, and to the members of the staff of the Institute factory who provided the grading reports on the cheese.

REFERENCES

(1) Spicer & Burgwald (1935). *Nat. Butter and Cheese J.* **26**, (21), 14.
(2) Price (1936). *J. Dairy Sci.* **19**, 185.
(3) Dolby, McDowall & McDowell (1937). *J. Dairy Res.* **8**, 74.
(4) *British Standard Methods for the Chemical Analysis of Cheese*, no. 770. 1938.
(5) Van Slyke & Bosworth (1907). *Tech. Bull. N.Y. St. agric. Exp. Sta.* no. 4.

(*MS. received for publication* 26 *June* 1940)

619.2:618.19-002.5

268. SUBCLINICAL STAPHYLOCOCCUS MASTITIS IN HERDS FREE FROM STREPTOCOCCUS MASTITIS, AND ITS EFFECT UPON MILK COMPOSITION

By P. M. F. SHATTOCK and E. C. V. MATTICK
National Institute for Research in Dairying, University of Reading

It is now well established that infections of the udder particularly with *Str. agalactiae* are of great economic importance in that the useful life of the dairy cow is shortened, quarters lost and milk yields diminished. It is not always realized that subclinical infections (*Str. agalactiae*) may seriously affect the composition of the milk, making for difficulty and loss of quality in the preparation of dairy products. Thus it has been shown by Foot & Shattock (1) that when the percentage of solids-not-fat in milk falls below 8·5, subclinical mastitis due to *Str. agalactiae* is present in a high percentage of cases. Similar conclusions were reached by Rowland & Zein-el-Dine (2,3). Foot & Shattock (1) also presented some evidence of the converse, i.e. that many cases of streptococcus mastitis result in milk low in solids-not-fat. In these experiments the samples were plated on Edwards's aesculin crystal violet blood agar which is designed partly to prevent the growth of other organisms, notably staphylococci. In our experience Edwards's medium is most successful in preventing the growth of staphylococci, and greatly facilitates the recognition of the streptococci for which it is used as a routine diagnostic medium. It is, of course, well recognized that clinical staphylococcus mastitis is not uncommon in this country, and Hopkirk (4) reports that staphylococcus mastitis is widespread in New Zealand. Infection with those strains which presumably produce toxin may, on becoming clinical, cause serious illness or death of the animal, but we have observed no such cases in the course of our work.

It is not so well recognized that staphylococci may give rise to subclinical infections whose effects on the composition of the milk may be similar to those produced by *Str. agalactiae*. Some workers have indeed accepted staphylococci as part of the "normal" udder flora, regarding udders in which they occur as normal.

Minett (5) and Plastridge *et al.* (6) have reviewed the extensive literature on the occurrence and bacteriology of staphylococci of the bovine udder, and we do not therefore propose to do so here.

Owing to the suppression of growth of staphylococci by crystal violet, it is not surprising that the true extent of subclinical or mild cases of staphylococcus infections in this country should be to some extent unrecognized. In the course of herd examinations of single quarter samples for the detection of *Str. agalactiae*, our fear that small numbers might be missed owing to the bacteriostatic effect of the dye or from mere failure to grow, led us to plate parallel samples from half the centrifuged deposit from 10 ml. of milk on aesculin blood agar without crystal violet. With milk from herds under our observation

proved by careful bacteriological tests to be free from *Str. agalactiae* infection, we have found that of 428 "cow tests", 92 or 21 % showed haemolytic staphylococci. There was no single case of severe or even patent mastitis due to staphylococci.

The herd of this Institute consisting of Shorthorn and Guernsey cows has also been under a mastitis control scheme (*Str. agalactiae*). Single quarter samples from individual cows were examined 10–14 days after calving, and before the animal was allowed to join the herd. Complete herd tests were carried out approximately every 6 months. At a time when it was known that no streptococcus infection existed, "clots" on the filter were reported from the dairy. On tracing these to their source and re-examining the milk of the offending cows, it was found that staphylococci but no streptococci were present. The nine cases of staphylococcus infection reported here (Table 2) were eventually discovered in this herd when the technique of plating on aesculin blood agar without crystal violet was used.

The routine use of cow blood in the bacteriological examinations was fortunate, as the presence of haemolytic staphylococci might otherwise have been completely missed. The necessity for the use of cow blood in the detection of staphylococcus mastitis has been stressed by Minett (5). It must be emphasized that the counts of *Staphylococcus aureus* recorded in Table 3 are mere approximations, and are under-estimates because the typical appearance is given only with surface colonies, haemolysis being partially or totally suppressed with colonies growing in the depth of the medium.

Because of our experience of the effects of subclinical streptococcus mastitis on the composition of the milk, chemical examinations were made on those single quarter samples which yielded staphylococci. When abnormalities of milk composition are found they may, we think, be taken as evidence that the staphylococci present had produced an actual udder infection and were not merely present as saprophytes. A chemical examination was accompanied by a bacteriological examination in each case, either on the same sample or within a few days. When *Staphylococcus aureus* was found it was identified by the following reactions at 37° C., which are summarized in Table 1.

Table 1. *Characters of staphylococci isolated*

Haemolysis production	+
Growth on Edwards's medium	−
Coagulation of rabbit plasma	+
Lipase (cream)	−
Utilization of $NH_4H_2PO_3$	−
Liquefaction of gelatin	+
Litmus milk	A.C. P.R.
V.P. test	+
Indol	−
Nitrate reduction	+
Catalase production	+
Pigment	Golden
Mannitol	A.
Dextrose	A.
Sucrose	A.
Lactose	A.
Maltose	A.
Raffinose	−
Salicin	−
Inulin	−

A. = acid. C. = clot. P.R. = partial reduction.

Characters of staphylococci isolated

Haemolysis on cow blood agar. Surface growth on 0·5% cow blood agar was observed after incubation at 37° C. for 24 hr., and again after standing at room temperature for a further 24 hr. Colonies gave typical clear zones surrounded by a well defined darker zone (Minett (5)).

Pigment. Golden.

Growth on Edwards's medium. None.

Soluble haemolysin production. Positive result by Bigger's method (7) using rabbit corpuscles.

Clotting of plasma. Using Walston's technique (8) rabbit plasma was clotted in 1½ hr. at 37° C.

Lipolysis. Strong on tributyrin agar but absent on agar with 0·2% sterile cream.

Utilization of $NH_4H_2PO_4$ as source of nitrogen. No growth.

Gelatin. Rapid liquefaction at 22° C.

Litmus milk. Acid and hard clot with partial reduction of litmus and some expression of whey.

Indol test. Negative.

Voges-Proskauer test. Variable.

Nitrate reduction. Positive.

Biochemical reactions. In mannitol, dextrose, sucrose, lactose and maltose, acid, no gas. In raffinose, inulin, salicin, no acid, no gas.

Catalase. Positive.

Clinical observations

With four cows only was any macroscopic abnormality of the milk or udder observed. The five remaining cows showed no clinical symptoms at any time.

Puppet. On calving showed a small quantity of blood in the milk of the R.H. quarter and, later, a few small clots were seen.

Winnie 6. Showed a little blood in the milk of both left quarters at calving.

Flora 31. The L.F. quarter was slightly swollen about 6 weeks before *Staphylococcus aureus* was demonstrated in this quarter.

Penelope 2 gave small clots in the milk 5 days after *Staphylococcus aureus* had been isolated.

In no instance did any of the slight *clinical* symptoms detailed above persist.

That *subclinical* staphylococcus infection may persist over many months and for more than one lactation is however shown in Table 2. Moreover, it

Table 2. *Persistency of staphylococcus infection*

Cow	Breed	Dates of births of calves over period of bacteriological testing	Date of first test	Date of last test	No. of bacteriological tests done	No. of positive tests
Puppet	Shorthorn	7. xi. 38 24. xii. 39	30. i. 39	7. iii. 40	7	7
Flora 31	,,	26. i. 39	15. ii. 39	28. viii. 39	5	5
Valencia 3	Guernsey	19. ix. 37	8. ii. 39	Sold	1	1
Penelope 2	Shorthorn	12. iii. 39	11. viii. 39	11. iii. 40	4	3
Clara 5	,,	9. vi. 39	28. viii. 39	7. iii. 40	3	3
Fanny 2	Guernsey	14. iv. 38 1. vi. 39	28. viii. 39	7. iii. 40	4	4
Flora 45	Shorthorn	7. xii. 38 8. i. 40	28. viii. 39	7. iii. 40	5	4
Rosalie 7	,,	17. v. 38 13. xii. 39	28. viii. 39	11. iii. 40	5	3
Winnie 6	,,	19. xi. 38 26. xii. 39	28. viii. 39	11. iii. 40	4	3

appears from Table 3 that haemolytic staphylococci may be present in a quarter without change or with little change in the chemical composition (Flora 45). In most cases, however, significant numbers of staphylococci are accompanied by changes in the milk as reflected by the decrease in the casein number, and by differences in the *p*H (determined by the glass electrode), which is always higher in the infected quarters.

Table 3. *pH and casein numbers in relation to bacteriological findings*

Cow	Date of chemical test	Quarter	*p*H	Casein number	Approximate count of *Staphylococcus aureus*	Date of bacteriological test
Puppet	3. ii. 39	L.F.	6·68	79·62	0	3. ii. 39
		R.F.	6·68	79·30	0	
		L.H.	6·69	79·14	0	
		R.H.	**6·82**	**71·48**	600	
	20. ii. 39	L.F. & L.H.	—	75·53	25	22. ii. 39
		R.F.	—	76·52	12	
		R.H.	—	**68·10**	>2000	
	30. iii. 39	L.F.	—	75·50	0	30. iii. 39
		R.F.	—	76·05	0	
		L.H.	—	76·45	0	
		R.H.	—	**67·66**	>2000	
		L.F.	—	—	20	9. i. 40
		R.F.	—	—	40	
		L.H.	—	—	30	
		R.H.	—	—	>2000	
Valencia 3	14. ii. 39	R.H.	—	**73·74**	150	8. ii. 39
	20. ii. 39	R.H.	6·83	**73·05**	—	
Flora 31	17. ii. 39	R.H., R.F. & L.H.	6·78	**71·63**	1000	15. ii. 39
		L.F.	**6·85**	77·29	500	
	24. iv. 39	L.F.	—	**72·95**	250	24. ii. 39
		R.F.	—	77·82	0	
		L.H.	—	77·89	0	
		R.H.	—	79·64	0	
	1. v. 39	L.F.	—	**70·41**	150	1. v. 39
		R.F.	—	77·34	0	
		L.H.	—	76·16	0	
		R.H.	—	76·83	0	
Clara 5	4. ix. 39	L.F.	**6·83**	**69·76**	50	30. viii. 39
		R.F.	6·64	75·02	0	
		L.H.	6·64	76·49	0	
		R.H.	6·64	77·13	0	
Flora 45	15. ix. 39	L.F.	**6·94**	**76·50**	0	15. ix. 39
		R.F.	6·82	79·64	0	
		L.H.	6·80	81·04	0	
		R.H.	6·80	80·11	50	
Fanny 2	29. ix. 39	L.F.	—	79·43	0	30. viii. 39
		R.F.	—	80·09	0	
		L.H.	—	80·67	0	
		R.H.	—	**77·38**	150	
Lottie 7	28. iv. 39	L.F.	—	77·27	*Str. agalactiae* infection	28. iv. 39
		R.F.	—	77·56		
		L.H.	—	77·31		
		R.H.	—	78·25		

The casein number is empirical and is affected by the technique used. Owing to a slight variation the normal casein numbers above are rather lower than those of Rowland & Zein-el-Dine but all are comparable amongst themselves.

It may be noted that in the case of Puppet, where the casein number was estimated on three different occasions over a period of nearly 2 months, the value decreased for each quarter as the infection established itself, being lowest in the R.H. quarter which excreted the largest number of staphylococci. In spite of the evident establishment of infection the passing of clots, already referred to under clinical observations, became less frequent and they were smaller in size.

With Flora 31 the L.F. quarter gave very low casein numbers, corresponding to the appearance of staphylococci in the milk from that quarter. Although the presence of these organisms was not recorded in the milks from the other quarters the casein numbers were below normal. This suggests that the organisms are excreted intermittently.

Flora 45 shows a normal casein number for the R.H. quarter associated with a small number of staphylococci, while a low casein number for the L.F. quarter was not associated with this organism. Four months later, however, at the beginning of the next lactation, small numbers of staphylococci were found in both left quarters.

Summary

In the milk of herds "free" from streptococcus infection, the presence of staphylococci has been shown to accompany chemical changes in the composition and abnormalities of *p*H.

In mastitis control schemes, based on *Str. agalactiae* infection, cases of staphylococcus infection are missed because of suppression of growth on the routine crystal violet blood agar of Edwards. Further, as clinical cases of staphylococcus mastitis causing serious disturbance are comparatively rare, the subclinical type may be present to a considerable extent and yet remain unsuspected. Thus in herds free from streptococcus mastitis, we have found that in 428 "cow tests" staphylococci were found in 21 %. No clinical case was observed, although careful records of all abnormalities of udders and milks were kept.

The data which we have obtained show that staphylococcus infection accompanies changes of chemical composition which may be of importance in the preparation of milk products, but before categorical statements can be made more observations are obviously necessary.

Our thanks are due to Dr A. T. R. Mattick for suggestions and continuous help with this investigation.

REFERENCES

(1) FOOT, A. S. & SHATTOCK, P. M. F. (1938). *J. Dairy Res.* **9**, 166.
(2) ROWLAND, S. J. & ZEIN-EL-DINE, M. (1938). *J. Dairy Res.* **9**, 175.
(3) —— —— (1938). *J. Dairy Res.* **9**, 182.
(4) HOPKIRK, C. S. M. (1938). *Rep. 1st Imp. Vet. Conf., Lond.*, 62; and personal communication.
(5) MINETT, F. C. (1937). *J. comp. Path.* **50**, 101.
(6) PLASTRIDGE, W. N,, ANDERSON, E. O., WILLIAMS, L. F. & WEIRETHER, F. J. (1939). *Bull. Storrs agric. Exp. Sta.* No. 231.
(7) BIGGER, J. W. (1933). *J. path. Bact.* **36**, 87.
(8) WALSTON, H. D. (1935). *J. Hyg., Camb.*, **35**, 549.

(*MS. received for publication* 13 *August* 1940)

BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION E. THE DISEASES OF DAIRY CATTLE

I. Mastitis
II. Contagious abortion
III. Tuberculosis

619.2:618.19–002.5

I. MASTITIS

Discussions on the general problem of mastitis and on its significance from both the economic and the public health aspects have been published by Udall (1), Minett (2), Stableforth (3), and in the *Report of the First Imperial Veterinary Conference* (4). The heavy economic loss due to mastitis is stressed by all the authors. There appears also to be very general agreement that the common cause of mastitis in Europe and America is infection with *Str. agalactiae* (*Str. mastiditis*, Lancefield group B). Hopkirk, however, states that in New Zealand staphylococcal and micrococcal mastitis are of equal importance. There is general agreement that infection via the teat is the usual method of infection and that management, hygiene and the handling of cows plays a great part in reducing losses due to mastitis. Heredity is also considered by some to be a factor of importance, as in hormonal imbalance. Stableforth (5) has published a general survey of mastitis in Britain in which he conservatively estimates the loss from this disease at £1,000,000 per annum. He discusses the diagnosis, treatment, prevention and control of the disease. Minett (6) has recently published a general article on the control of mastitis with special reference to a herd he has not been able to free from the disease.

General

In an official report from Australia (7) a summary is given of the results obtained in the examination of fifty heifers coming into milk for the first time. *Str. agalactiae* was recovered from sixteen quarters during the first week but did not persist, while 50 % of the quarters remained sterile during the whole lactation. The other 50 % gave high bacterial counts either occasionally or persistently, usually due to micrococci, but staphylococci were present in fourteen quarters and *Str. agalactiae* in nine. In the second lactation 34 % of the quarters remained sterile while high counts in the other quarters were due mainly to micrococci, staphylococci being present in twenty-two and *Str. agalactiae* in two. It was noted that in both lactations infections due to this latter organism were transient and usually did not cause high counts. Clinical mastitis was found twice during this period. Streptococci other than group B were also isolated, including many group II infections, but these did not persist nor did they produce mastitis. During the two lactations thirty-nine quarters showed haemolytic staphylococcal infections and twelve developed clinical mastitis. Micrococci were found to cause persistent infection in many cases, while those quarters which remained free from infections consistently had cell counts of less than 100,000 per ml. *Str. agalactiae* was recovered from the faeces of seven cows and from sores on the teats of two. Clots were frequently present in the milk from sterile quarters. Klimmer & Haupt (8) tested the virulence of *Str. agalactiae* from clinically normal cows. Their method was to fasten small bags of saccharose chalk cultures to the teats

every night and to leave on for about a week. Three strains were used. One strain failed to infect, another infected the cow in which the first had failed, and the third infected the cow on which it was tried. All three strains failed, however, to infect a third cow which was subsequently successfully infected with the same technique by using a strain from a clinical case. They conclude that the strains from the non-clinical cases are of less virulence than those from clinical cases. In connexion with the histopathological conditions produced by the disease, Schalm & Haring (9) have described a method of preparing for histological examination the whole udder by injection of formalin. When required, serial sections can be cut by a meat slicer. Swett *et al.* (10) have noted that, in a series of cases in which the animal as a calf or young heifer had discharged pus from a teat, the quarter became deficient after parturition and often blind. They considered that this was not always due to direct damage to glandular tissue but rather to occlusion of the teat leading to involution of the gland.

Peterson & Hastings (11) have emphasized their view that primary lesions due to "non-specific" affections of the udder are necessary before *Str. agalactiae* can become established under natural conditions. In support of this hypothesis they quote their pathological findings in a series of affected cows and state that in ten cases with abnormal udders free from *Str. agalactiae*, permanent infection with this organism was easily produced by the insertion into the teat canal of glass rods previously dipped in broth cultures. Bryan (12) was also able to produce experimental mastitis by dipping injured teats into cultures. The same cultures injected intravenously, subcutaneously, or fed did not cause infection of the udder. This author in conjunction with Taylor (13) also analysed his results on 121 cows and found that there was no significant difference between the rate of infection in fore and hind quarters or right and left sides. Johnson (14), however, considered from his studies of the structure of the teat that pressure on distended quarters (especially the hind) may overcome the natural resistance of the teats to infection. He found that in some teats the lining membrane showed pouches or folds and projected around the lactiferous sinus opening in the form of a rosette. While milk was shown to be forced back into the sinus during milking it was found that carbon particles tended to remain on the pouches, folds or rosette, and were not removed by milking. He suggested that bacteria might do the same and hence that a teat having few irregularities would be less likely to be infected by invading bacteria. This type of teat may be inherited. Ward (15) studied the mastitis records of 709 daughter-dam pairs. There was evidence of an hereditary factor in the susceptibility to mastitis, but unfortunately no bacterial tests were performed, the diagnosis of mastitis resting entirely on the examination of gravity cream for leucocytes (Hopkirk's method (4)). The cells in milk vary considerably according to the period of lactation and Rönnefahrt (16) considers that no microscopical change during the immediate pre- and post-parturient periods is of any value as a guide to abnormality since great variations normally occur at this time. Little (17) also considers that mastitis is correlated with the anatomical and functional characters of the teat. He advances the theory that not only damaged teats but also teats over-stimulated by prolonged lactation or where muscular atony of the sphincter has occurred are particularly liable to lead to mastitis. He does not hold, however, that the resistance offered by the udder to infection is purely mechanical, but considers that infection even after entering the teat may be controlled by a bacteriostatic substance present in the milk which either destroys or immobilizes the bacteria so that they are expelled at the next milking.

Meigs *et al.* (18) studied the effect of introducing a machine into a hand-milked herd. The herd was divided into three groups. The first group continued to be milked by hand and remained normal. The second, which was milked by the machine at low

vacuum and hand stripped, did not show mastitis though the leucocyte count and chlorine content were increased. The third group was milked by machine using high pressure and stripped while the machine was still on. These showed a rapid rise in the leucocyte count, a reduction of yield and an increase in the chlorides and bacteria followed by acute mastitis. When hand milked again these animals recovered. It appears from these experiments that mechanical injury is probably done to the teat by the milking machine if the vacuum is too high or maintained too long.

Little's view that bacteriostatic substances may be present in the milk is borne out by further work by the same authors (Burkey *et al.*(19)), in which they showed that the germicidal properties increased in direct relationship to the disease until the acute stage, when they decreased. Blood serum inhibited the growth of *Str. agalactiae* in milk from the same cow but not in milk from another cow. This inhibitory effect disappeared if the serum was stored for four days or if it was from a cow suffering from acute mastitis.

The theory held by many farmers that high protein feeding increases the liability to mastitis was tested by Stang *et al.*(20) on three groups of six, five and six cows respectively. They found that in group 1 (high protein) three showed clinical and one latent mastitis; in group 2 (medium protein) all were infected (four clinically) and in group 3 (normal diet) one latent and one clinical case occurred. Luyken(21) obtained practically identical results. In group 1 (six animals on high protein) three developed infection within 6 months, in group 2 (five animals on medium protein) all five showed infection, while of the six animals in group 3 on normal diet two showed infections. There was thus in neither case any significant difference between the high and low protein diets.

Miller & Johnson(22) correlated their bacteriological findings with clinical observations, grouping the udders into Udall's four groups(23) and considered with Udall that this grouping is valuable as a control measure. However, their own figures do not bear out this conclusion. If one groups together their class 1 and 2 udders, i.e. those with no or very little induration, it is seen that they comprise 60 infected and 134 non-infected cows. Udall's grouping would, therefore, leave 60 of the infected animals in the clean herd. Further, their class 3 and 4 udders, comprising cows with definite induration or clinical mastitis (i.e. those to be disposed of) consisted of 231 infected and no less than 192 non-infected cows. This proportion of non-affected cows with indurated udders appears to be very high and would lead to unnecessary loss. This work followed Miller's earlier work(24) on the histopathology of infected udders of cows condemned for advanced clinical mastitis. The changes were varied but not very marked. Out of 1216 quarters only twelve contained purulent foci. The usual change observed was that the inter-alveolar tissue had become hypertrophied and was filled with fibroblasts, round cells, polymorphs and a few eosinophiles. Signs of acute inflammation were rarely seen. A paper by Peterson, *et al.*(25) describes similar findings, except that few neutrophil leucocytes were found in cases of mastitis in which they were unable to demonstrate any pathological organism except staphylococci. These latter were in such small numbers that they were not considered to be the cause of the mastitis, which was thought to be due to a filterable virus. There seems to be no necessity to assume the presence of such a virus since the changes described are such as would be expected in a low grade staphylococcal infection.

Sherman & Niven(26) studied the haemolytic streptococcal flora of 68 raw and 245 pasteurized samples of commercial milk. Typical narrow zone B-haemolytic *Str. agalactiae* were usually found in the raw milk samples, but not in the pasteurized. Group C streptococci, varying in number from a few to many thousands per ml. and usually showing a broad zone of haemolysis, were also isolated from raw milk. The

only haemolytic streptococci found in pasteurized milk (and then only in small numbers) were the heat resistant group D type (durans and zymogenes) which occurred in 8·5 % of the samples. Bendixen (27) also found that the majority of raw market milks in Copenhagen contained B-haemolytic streptococci varying in number from 100 to 40,000 per ml. In examining quarter samples 1028 strains of streptococci were studied; the majority were group B (*Str. agalactiae*). About one-third of the strains were, however, biochemically similar to the British group 2. Group C strains were also sometimes found, usually being associated with sporadic clinical mastitis. Enzootics were observed in certain herds, while latent or subclinical cases were also found. Experimentally, mastitis could be induced in cows by introducing human double zone B-haemolytic streptococci into the udder via the teat. Eleven out of twelve quarters so treated by Little (28) became infected but mainly cleaned up within varying short periods.

Little has shown (29) that the British group 2 strains (*Str. dysgalactiae*) are serologically related to Lancefield's group C, being capable of precipitating with, and removing all group antibodies from, group C sera. The converse is also true (i.e. group C antigen precipitates with group 2 antisera). Plastridge has informed this author that his *Str. pseudo-agalactiae* is identical with group C (British group 2 *Str. dysgalactiae*). Little (30) has also drawn attention to the importance of group 3 (Minett) streptococci. In one herd of only nineteen cows in which *Str. agalactiae* had been eliminated by the sale of all infected cows, eight cases of group 3 infection occurred. In only one case was the milk visibly altered but induration of the udder was common. He also comments on the fact that sale of the affected animals was sufficient to prevent any spread of the disease due to *Str. agalactiae*, and concludes that this is evidence in favour of the bovine udder being the only habitat of the organism. Brown (31) has studied the relationships between serological type and biochemical reaction of β-haemolytic streptococci. Over 1000 strains were examined, and it was found that the reactions in sorbitol and trehalose served as a rough guide to the groups. Group B mastitis streptococci were sorbitol negative and could be further distinguished by the use of the hippurate broth test. All animal strains fermented lactose and all human strains salicin. 40 % of the animal strains failed to ferment the latter, and 66 % of the human strains failed to ferment the former. Stewart (32) found that some group B streptococci gave an appearance of capsulation when stained by Lyon's technique. There was no correlation between this and any other characteristic and, since both Muir's and Hiss's methods failed to yield similar results, he concluded that no capsules were actually present and that their apparent presence was due to some other factor.

Davis & Rogers (33) have studied the reaction of the common milk streptococci in blood agar. They draw attention to the difficulty of deciding between "weak β" and β, and consider that only true clear zones should be described as β. They also advocate the use of the term "bleached" to describe the condition in which the area around the colony is colourless but contains cellular stroma.

Diagnosis and control

The usual method of diagnosis for contagious mastitis continues to be by direct cultivation of the milk in Edwards's blood-agar aesculin plates (4). Recently Mészáros (34) has described a selective medium, which appears to be a modification of that of Klimmer *et al.* (35), consisting of an agar base with brom-cresol purple saccharose and "serum-alkali albumen". The streptococcal colonies show yellow. An advantage claimed for the medium is that it will keep for considerable periods (up to 4 months) without deterioration. A further medium for the isolation of *Str. agalactiae*

has been devised by Edwards (36). This is a glucose broth medium containing crystal violet (1:1,000,000) and sodium azide (1:10,000). Edwards found that the results obtained agreed with those of the blood agar plates in 96 % of the 1014 quarter samples and in 93 % of 342 mixed samples. Later work by the present reviewer (unpublished) showed, however, that though similar results were obtained as long as the batch of peptone used by Edwards was available, fresh batches of peptone have not always given such clear cut results.

Plastridge *et al.* (37) describe their methods for the laboratory diagnosis of mastitis as follows: (i) presumptive tests, i.e. appearance of sample, brom-thymol-blue test, and leucocyte count, (ii) incubation of 10 c.c. samples at 37° C. for 16–20 hours, (iii) microscopic examination for streptococci, and (iv) surface plating on crystal violet aesculin agar of all samples containing streptococci or which were positive to the presumptive tests. In the latter case plain blood agar (to detect *C. pyogenes*, staphylococci and *Bact. coli*) is also used. After incubation for 24–48 hr. selected colonies are picked, especially when *Str. agalactiae*-like colonies are present in samples which were negative to the presumptive tests or from previously clean cows. These authors give the results in five classes: class 1 negative, class 2 positive to presumptive tests but showing no streptococci, class 3 positive to presumptive tests but showing streptococci other than *Str. agalactiae*, class 4 negative to the presumptive tests but showing *Str. agalactiae* or *pseudo-agalactiae*, and class 5 positive to presumptive tests with *Str. agalactiae* present. Davis *et al.* (38) state that their routine method of diagnosis is as follows: brom-cresol-purple paper tests are taken on the fore-milk and sample collected from the early mid-milk are plated immediately on returning to the laboratory on crystal violet aesculin blood agar, plain blood agar, and milk agar. Obvious *Str. agalactiae* colonies are noted; others suspected to be this organism are picked off and incubated in yeast dextrose litmus milk. This test is used as the biochemical criterion for *Str. agalactiae*. Each cow is tested twice at an interval of not less than 10 days. Davis *et al.* were mainly concerned with the differentiation of the causal organism, and note that *Str. bovis* is the most difficult to distinguish from *Str. agalactiae*. They consider Edwards's medium is not entirely satisfactory if the true condition of the udder flora is required, as staphylococci are inhibited as also are (according to private communications made to them) group 3 (Minett) and *Corynebact. pyogenes*. They mention the results of 256 tests in which one or more of three tests on different days was positive. Of 124 strongly positive at one test, 42 were negative at another; of 64 positive at one test, 23 were negative at a further test; and of 68 with a few colonies of *Str. agalactiae* at one test, 34 were negative when retested. They consider the significance of small numbers of organisms in otherwise normal udders as obscure, but class the cows as positive in their eradication scheme.

Williams (39) also describes his method for the detection of mastitis streptococci in milk. He points out that routine clinical examinations of cows, together with chemical tests, do not detect all the infected cows. He advises the use of cultural tests on all samples in which microscopical examination has shown long-chained streptococci or high cell content. If positive, individual samples are taken and any haemolytic streptococci found are removed and tested in sodium hippurate and aesculin broths. Davis & Rogers (40) have described a simple confirmatory medium for *Str. agalactiae* which consists of yeast litmus milk to which aesculin and ferric chloride have been added. Acid and clot without reduction or darkening of medium is taken as presumptive evidence of *Str. agalactiae*. The only other streptococci with which it may be confused are certain non-aesculin-fermenting strains of *Str. pyogenes*. Foot & Shattock (41) have recorded that 70 % of the cows found by them to be low in solids-not-fat were suffering from mastitis, while Rowland & Zein-el-Dine (42) found

that 88 % of their samples with a value lower than 8·8 were infected with mastitis. The casein number test has been described by these authors (43) who studied the effect of mastitis on this index of normality, and they found that the normal figure of about 78 is lowered in mastitis. Their results for the chemical and bacteriological tests disagreed in 8·6 % of cases, and they also found that the casein number was not reliable with cows advanced in lactation. It was necessary to perform the tests within a few hours of taking the samples unless they were stored at 0° C. with added preservative such as formalin or mercuric chloride.

Rowland & Zein-el-Dine (44) have also made a statistical examination of the agreement between the indirect tests and the bacteriological findings. Using the method of the mean square contingency where 1 represents complete agreement and 0 complete disagreement, they found that the correlation was: casein number, 0·698; chloride, 0·314; centrifuge deposit, 0·335; solids-not-fat, 0·314; brom-cresol-purple, 0·218; brom-thymol-blue, 0·085; presence of cells, 0·061; catalase content, 0·022.

Johns & Hastings (45) examined samples taken at consecutive milkings to determine the reliability of the chloride, catalase, and *p*H tests for detecting contagious mastitis. As infected quarters were found on many occasions to yield normal milk and healthy quarters to give milk of abnormal reaction which may fluctuate widely at different milkings, they do not consider these tests as satisfactory for diagnosing mastitis. Of the three the catalase is the most sensitive. In the chloride tests Sanders (46) suggests that any values over 0·12 % are suspicious, and all over 0·15 % positive. Little (47) has published the results of the examination of the fore-milk from thirty-one quarters of eight first calf heifers before and after experimental infection. The *p*H and chloride tests, the leucocyte count, and plating in deep blood agar were used. In 1010 daily examinations made before infection most of the results fell within the limits generally accepted for normal milk (*p*H 6·6; chlorides, 0·135 %; leucocytes, 3×10^5 per ml.). After experimental infection 2163 daily samples were tested. The plating test gave 100 % correct results and the leucocyte count 94 %, while the *p*H and chloride tests were only 50 % correct. Fay *et al.* (48) studied the brom-thymol-blue test, the leucocyte test and the microscopic examination of incubated samples of milk on 112 cows. These were divided into three classes: A, free animals; B, suspicious, i.e. high leucocyte count (over 5×10^5 per ml.); C, positive, i.e. cows with streptococci in incubated milk samples, usually associated with high leucocyte counts. They compared the results of the tests and found that a positive brom-thymol-blue test is good presumptive evidence of mastitis, but that false negatives are very numerous. Leucocyte counts above 5×10^5 per ml. were found in only 33 % of the samples showing streptococci. It was also found that in classes B and C, cows, even if they cleared themselves of infection, usually reverted to positive. Kretchmar (49) also examined the value of brom-cresol-purple and brom-thymol-blue tests and found them unsatisfactory. Davis & McClemont (50) compared twelve common methods of detecting mastitis, and decided that none alone nor any combination of them would be of value. McClemont & Davis (51) stated that in a series of examinations over a long period, only rarely was a sample with a total plate count of less than 100 encountered in cases of mastitis, and concluded that this might be of value in eradication schemes. They pointed out, however, that a sterile plate or one with a very low count should be regarded with suspicion, as it may indicate severe mastitis. They considered that only very rarely do uninfected cows give a count of over 1000. The same authors (52) stated that, though the methylene blue reduction test was delayed by the presence of mastitis, this was of no value for detecting the disease.

McCulloch & Fuller (53) found that the Hotis test, though possibly valuable in herds where *Str. agalactiae* is practically the only organism present, is of less general

value. They found that any streptococci give the reaction if they (i) stimulate the production of agglutinins, (ii) grow in the presence of 0·025 % brom-cresol-purple in a milk medium, (iii) form clumps on the side of the tube when grown in the presence of their agglutinins, and (iv) produce sufficient acid from lactose to increase the hydrogen-ion concentration to about *p*H 5·4. Gwatkin *et al.*(54) examined the milk from 594 cows by the brom-thymol-blue, rennet, microscopic and bacteriological tests; 156 showed streptococcal infection. The brom-thymol-blue and rennet tests combined failed to detect 25·5 % of cases and clinical examination failed in 20·7 %. Fay *et al.*(48) made a comparative study of the brom-thymol-blue, leucocyte and microscopic tests for mastitis. They examined 7000 samples and found that the brom-thymol-blue test detected only 21·1 % of the infected samples and that the leucocyte test (over 500,000 per ml.) showed only 36·7 % of infected samples. The bacteriological test failed to reveal 14·7 % of the samples known to be infected.

The application of measures for controlling contagious mastitis has spread considerably in recent years, especially in the United States, Switzerland and Britain.

Udall *et al.*(55) have described their methods for the control of mastitis in New York State. They consider that complete eradication is impossible in most herds, and they aim rather at the control of the spread of the infection. Their method is to examine all udders and to classify these into four classes, i.e. class 1, excellent; class 2, normal; class 3, fair; and class 4, bad (advanced fibrosis due to chronic mastitis). At first they examined the udders bacteriologically to determine the extent of infection in each class. They found practically none in classes 1 and 2, a medium rate of infection in class 3 and a high rate in 4. They considered the latter to be the chief source of infection, and that their removal would have a restraining influence on the spread of the disease. The cows are milked in the order class 1, 2, 3, 4, and should any in classes 1 or 2 show abnormality the milk is examined by the strip cup. If necessary further examination is made by the brom-thymol-blue test, the field test for chlorine, the milk incubation test or by bacteriological examination. Any early cases shown by these tests are treated by hourly milking, hot magnesium sulphate packs and reduction of the grain ration. As in any control programme, the authors give the farmer advice with regard to hygiene, disinfection, etc., and advocate washing the hands, udders and teats with a chlorine preparation. These methods are claimed to have been applied successfully in practice.

Horwood & Bryan(56) applied a somewhat different technique in their control of mastitis in a single herd. They examined the milk bacteriologically and removed the affected cows. They then bred from a bull whose progeny had smaller and less pendulous udders than those previously normal in the herd. The usual hygienic measures were adopted and, whether these were the cause or whether the small udder advocated had some effect, only two cows showed streptococci in five years. It is stated that these were treated with a lacto-vaccine consisting of 30 ml. of infected milk with 0·3 % brilliant green, which was successful in clearing up the disease and eliminating the streptococci. At the general discussion at the Imperial Veterinary Conference(4), it was suggested that hereditary susceptibility might be dependent on anatomical peculiarities, and Datta mentioned the possibility of hormonal imbalance being an important factor. Emphasis was laid on the importance of maintaining good hygienic precautions and of handling teats carefully, especially noting that any sores and abrasions should be treated promptly. With regard to diagnosis, it was considered that a relatively simple method of accurate diagnosis of streptococcal mastitis was a combination of cultural and serological methods using a Lancefield group B precipitating serum. In this connexion an article by Brown(57) may be noted. He has described a method of carrying out precipitation tests, using petri dishes and hanging drops, and claims that this is both easy to read and very economical in use.

Steck has given a full review of the methods he uses in the state control of mastitis in Switzerland (58). During the last ten years a systematic State-aided campaign has been drawn up. Diagnosis is made in the laboratory, using cultural methods, while treatment is in the hands of practical veterinary surgeons using injections of "zysternol", the English equivalent of which is euflavine. In the county of Berne sixty-five herds have been tested and treated by these methods. It seems that most of the herds were small, only about 600 cows in all being included. The original infection rate was 40 %. At the second test this rate was reduced to less than 8 %, in twenty-eight herds no infection was present, and in a further eleven the only infected cows revealed had been treated or removed. In fourteen other herds the extent of infection had been greatly reduced (by at least one-half). Kästli (59) also reported the results of a year of mastitis control work in Switzerland. 1172 cows in 106 herds were included and 389 were found infected. Within a year sixty-seven herds were free, though 17 % of the infected animals had been slaughtered. The others were treated by Steck's method of udder infusion. The difficulties found were: the unwillingness of certain owners to allow treatment of cows which only showed latent infections, the drying up of quarters because the milkers did not realize that the clinical signs immediately following treatment would be cleaned up by adequate stripping, the failure of owners to understand that latent cases are contagious, the use of unsatisfactory methods of treatment by veterinary surgeons, difficulties in transit of milk samples causing unsatisfactory diagnosis and lack of control samples after treatment.

Klimmer & Haupt (60) attempted to free a herd of sixty cows in which 70 % were infected. They had not succeeded after three years. They report that fore-quarters were more often affected than hind, and that of forty-nine heifers seven showed infection in the first test. Though new infections showed no marked seasonal incidence, they occurred more often towards the end of lactation.

Treatment

Giebner (61) compared the efficacy of trypaflavine, entozon, rivanol, prontosil, and a nitro-acridine derivative on *Str. agalactiae in vitro*. The results were assessed by a mean value "based on the time in minutes taken to reduce the original count to a prearranged extent with allowance for the time before any effect was noticed and the time taken for complete sterilisation". In addition to broth as diluent he tested serum, mastitis secretion, normal milk and the secretion of a dry cow. The trypaflavine was found to be the most active and prontosil the least. Klimmer & Haupt (60) treated 254 quarters in milk and 66 dry quarters with entozon, using three negative tests as the criterion of cure. They found that 31·5 of the milking and 47·3 % of the dry quarters were cured, and 10·7 and 33·7 % respectively were probably cured. They note that many of the successes were in clinical cases. The importance of the actual technique of injection is emphasized by Steck (62) and Seelemann & Siemonsen (63). These authors disagree on the results obtained by euflavine injection as compared with entozon. Steck advocates euflavine, and considers that Seelemann & Siemonsen's bad results were due to performing the injections by the usual entozon method and not following the technique evolved by Steck, i.e. either injection in saccharose solution or by "cistern infusion". Stableforth & Scorgie (64) tested entozon and acriflavine at dilutions of 1/1250 and 1/10,000 respectively. With the former, cures were obtained in nine out of nine experimentally infected quarters, in forty-three out of sixty quarters with latent mastitis and thirteen out of thirty-seven clinically affected quarters. Acriflavine cured twenty-six out of twenty-nine quarters with latent infection, and nine out of fourteen clinically affected quarters. Gerstner (65)

infused dry and lactating cows with entozon or euflavine, the quarters being injected to full milking tension. The solutions were allowed to remain in the udder for 3 min. if lactating, or 9–24 hr. if dry. Of seventy-six quarters treated, seventy-four were cured. Euflavine was the more successful, and the degree of infection did not affect the ultimate result. Several infusions were often given.

The effect of sulphanilamide has been examined by many authors. Gay *et al.* (66) have studied the action of sulphanilamide on streptococci *in vitro* and *in vivo*. They found that the bacteriostatic power of the drug *in vitro* is regulated, as with most of these agents, by the nutritive value of the medium. The more nutritive the medium the less the inhibitory power of the drug. It does not destroy the bacteria nor impair their virulence, but does inhibit their growth *in vivo* and also, under certain conditions, temporarily inhibits haemotoxin formation. It is not absorbed by packed cells. For the treatment of mastitis Allott (67) recommends doses of 30 g. of sulphanilamide four times a day, and considers that though the streptococci were not destroyed, there was definite improvement as judged by clinical and laboratory tests. Gildow *et al.* (68) found that a concentration of 8 mg. per 100 ml. of milk could be obtained by a dose of 10 g. per 100 lb. body weight. Of sixteen cows treated in various stages of infection none was cleared, though clinical conditions were improved. Intolerance of the drug by individual cows was marked, and one died of enteritis. Miller *et al.* (69) studied the concentration of the drug in the blood and milk, and found that a concentration of 15 mg. per 100 ml. blood could be produced by giving a dose of 0·4 g. per kg. body weight every 12 hr. by stomach pump. When given in food, two animals receiving 60 g. every 12 hr. refused the seventh dose, while two receiving 90 g. refused the third dose. The same authors further studied the effect of doses of 0·4 g. per kg. followed by 0·2 g. every 12 hours for six doses, then 0·14 g. for two doses and 0·1 g. for three doses. The concentration of sulphanilamide in the blood and milk was 20 mg. per 100 ml., and led to the temporary removal of the organisms from the milk. They recurred later, however. Hoge *et al.* (70) studied the effect of sulphanilamide on the number of mastitis streptococci and of leucocytes in milk, the drug being given in gelatin capsules in doses of 5–15 g. per 100 lb. body weight at intervals of 12 or 18 hr., and for periods of about 10 days. A dose of 15 g. was not well tolerated and had to be reduced, but a concentration of 8 mg. per 100 ml. of blood was obtained. The streptococci disappeared while the animals were under treatment, but recurred later. The effect on the numbers of leucocytes was variable. Little (71) treated five cows with mastitis with varying doses at various time intervals. He found that the number of organisms was greatly reduced during treatment, but that they were not eliminated. Bauer & Gunderson (72) also found that the numbers of the organisms in the milk were reduced so long as the drug could be detected in the milk, but that when it was no longer present the numbers rose again.

Stableforth (73) states that the peak concentration in milk is reached an hour or two later than in blood, but that it is maintained longer. After a dose of 1 g. per 5 lb. body weight *per os*, the milk contains 13 mg. % at 8 hr., 8 mg. % after 24 hr. and 2 mg. after 48 hr. After half this dose the values were 8 mg. after 8 hr. and 5 mg. after 24 hr. For the treatment of mastitis he recommends an initial dose of 1 g. per 5 lb. body weight or 1 g. per 10 lb., followed by dosing every 8 hr. by 1 g. per 30 lb. He states that clinical symptoms are usually improved, and that in just under 50 % bacterial cures have been obtained. The following further observations have been furnished by Dr Stableforth in a private communication:

1. Cows given *per os* three doses of 6 drachms per cwt. at intervals of 2 days. Of forty-seven cows (in five herds), three were cured (6·4 %).

2. Cows given *per os* a first dose of 6 drachms per cwt. followed by a further five doses of 3 drachms per cwt. at daily intervals. Of thirty-six cows (in four herds), eight were cured (22·2 %).

3. Cows given *per os* a first dose of 6 drachms per cwt., followed by further doses of 1 drachm per cwt. at 8 hr. intervals for 5 days. Of twenty-three cows (in two herds), eleven were cured (47·8 %).

4. Cows given *per os* one dose of sulphanilamide, 6 drachms per cwt., followed next day by udder infusion with a saturated solution of sulphanilamide (0·7–0·8 %). Of twenty-nine cows (in two herds), seven were cured (24·1 %).

5. Cows given *per os* one dose of sulphanilamide, 6 drachms per cwt., followed next day by udder infusion with acriflavine 1:10,000. Of twenty-eight cows (in two herds), thirteen were cured (46·4 %).

Twenty cows, which failed to respond to one or other of methods 3, 4 or 5, have been re-treated by the same or other method, about half being cured.

Observations on the value of a saturated solution of sulphanilamide as an udder injection showed that, though quite non-irritant, it was definitely less effective than the acridine dyes. Further, that combination of a single large dose of sulphanilamide *per os* with sulphanilamide infusion of the udder on the following day, was of less value than the combination of sulphanilamide *per os* and infusion with an acridine dye the following day. The last method was successful in several cases where sulphanilamide *per os* and udder injection with acriflavine had both failed when used alone.

Other types of mastitis

There have been few publications except on general lines on infections other than *Str. agalactiae*. Hopkirk (4) emphasized the importance of staphylococci infections in New Zealand. Miller & Johnson (22) found that forty-six out of 276 cultures of streptococci causing mastitis were not *Str. agalactiae*. Plastridge *et al.* (74) have studied the staphylococcal form of infection. They conclude that this is usually due to haemolytic staphylococci, that it is milder than that due to *Str. agalactiae*, that about 25 % of cases recover spontaneously, that the source is outside the udder, and that it is not possible to control its spread by segregation measures. A full description of the biochemical reactions of the staphylococci is given. Gwatkin *et al.* (54) found fifty staphylococcus, three *Corynebact. pyogenes* and ten *coli-aerogenes* infections in 594 cows. The coli infections were all acute and six ended fatally. Bendixen & Minett (75) have reported two cases where there was infection of cows by *Str. pyogenes* leading to outbreaks of streptococcal disease in man. In both cases the infection had a latent period when there was no sign of the disease, though streptococci were present.

619.2:616.999.61

II. CONTAGIOUS ABORTION

Serology

The production of an endo-antigen from *Br. abortus* comprising some 25 % of the cell has been described by Pennell & Huddleson (76). They state that this substance is toxic to guinea-pigs but that, if acetylated, its toxicity is destroyed while its antigenic powers are increased. Sera were obtained which precipitated to a dilution of $1{\cdot}5 \times 10^6$ when tested against the antigen. Guinea-pigs were not immunized by injection of endo-antigen, but in cattle and man injection resulted in high opsonic titres. Priestley (77) has concluded that only heat stable antigens are concerned with immunity, as he was not able to demonstrate any labile antigen by absorbing immune sera with heat treated suspensions and putting up the absorbed sera with living *Brucella*. A full study of the antigenic surface of *Br. abortus* and the chemical composition of its antigens has been published by Miles (78) and Miles & Pirie (79). They confirm the conclusion that the surface consists of A and M (*abortus* and *melitensis*) antigens and consider the proportion to vary from 50:1 to 10:1. One

conclusion of interest is that the rabbit when injected not only produces anti-A and anti-M agglutinins but also an anti-AM bearing receptors to both antigens.

Common & Kerr (80) have shown that the globulin content of the plasma rises in abortion. They found an increase in the globulin fraction of vaccinated and naturally infected cattle which was associated with the rise in agglutinin titre. Meyer & Huddleson (81) state that agglutinins may be absent from the blood even when *Br. abortus* and specific antibodies are present in the milk. They also state that the organism appears in the milk and may be present in the udder for a long time without agglutinins being demonstrable. Scrivner (82) studied the infection in the udders of cows in affected herds and in animals vaccinated as calves (strain 19). He concluded that *Br. abortus* is only found in the milk immediately after calving except where the blood titre is high enough to be diagnostic, and that, though vaccination prevents the udder from becoming permanently affected, vaccinated animals may secrete the organism after their first calving (in five out of twenty-eight of his cases). The strain excreted was not, however, the vaccine strain.

The rapid slide agglutination test with milk, though useful as a field agent, has been shown by Ehrlich (83) to agree with the tube agglutination test, using blood serum in only 74 % of 1044 comparative tests. If the test is used, Ehrlich advocates 5 % saline as the diluent and the use of thick dye-impregnated suspensions. Hermann (84) also advocates the use of haemotoxylin-stained emulsions for the rapid agglutination test and draws attention to an important practical detail, i.e. that the amount of stain must not be in excess of that required to stain the bacterial bodies. Any such excess remains free and, if used with milk, stains the casein clumps. In 23,900 samples he found that the rapid slide test corresponded well with the ordinary tube method. Thumann (85), however, found the rapid test to be unreliable when the tube agglutination titre was under 1/100. Rudolf (86) investigated a number of instances in which the rapid test and the tube test had not agreed, and concluded that with due care false positives to the rapid test could be avoided, but that false negatives could not. Seelemann & Wolf (87) made an extensive investigation into the value of the rapid slide test, and decided that it was very accurate and could be used in eradication schemes. Glässer (88) has shown that deposits found in his tests were not due to agglutination of *Brucella* but to the growth of an aerobic spore-former which was not inhibited by the 0·5 % phenol used as preservative. By substituting 0·01 % chinosol for the phenol, the organism was inhibited. Klimmer & Vogel (89) advocate the addition of saponin to the antigen, as this tends to reduce non-specific flocculation. Love (90) suggests using a 72 hr. liver-agar growth suspended in carbol saline and standardized so that 0·5 ml. yields a centrifugal deposit of 0·5 in. in a Hopkins tube. Elder (91) compared the plate and tube agglutination tests, and found the former to be slightly more accurate. The greatest differences arose with those sera whose titre was about 1:50. He considers that only 1:100 is positive, and that complete agglutination at 1:25 is negative and due to natural antibodies. This is in direct contradiction to Timoney (92) who considers any agglutination as probably positive and doubts the existence of natural antibodies.

Fitch *et al.* (93) have studied eighty-six suspicious reactors, i.e. those whose titre was less than 1:100. Monthly blood tests and fortnightly examinations of the milk by guinea-pig inoculation and culture were made. Placentas were also examined, and in ten cases post-mortem examinations were also made. Three became positive (i.e. over 1:100) and aborted. They were positive before aborting and could thus be removed from the herd. None of the negative in-contact animals became affected, so the authors have concluded that suspicious reactors are not dangerous in a herd. This may be true where monthly examinations are possible, but it appears to be an unwise procedure in normal herds. According to Fröhle (94) the agglutination test

itself is of less value than flocculation tests in detecting positive animals. Of 856 cattle which he considered to be positive by the results of all the tests, only 46 % were positive to the agglutination test, against 90 % to the Sachweh, 56 % to the Meinicke and 59 % to the complement-fixation tests. The validity of these conclusions is questionable since there is no evidence, other than the tests themselves, that these animals were actually affected. On the basis of a considerable number of tests Seelemann & Pfeffer (95), however, also consider the Meinicke and Sachweh tests eminently suitable for diagnosis, using either blood serum, whole milk or whey.

The ring test in milk has been further elaborated by Meinicke (96) who has described modifications of the flocculation test. In the first paper he gives a method of determining the optimum amount of antigen to use and introduces an alkaline broth control. In the second he states that centrifuging can be omitted if the tubes are left overnight at room temperature. Hermann (97) and Fleischhauer & Canić (98) have also modified the test. The former advocates the use of haematoxylin to colour the emulsion, while the latter describes the results of centrifuging the tubes to throw down the precipitate. If negative, the deposit is diffuse; if positive, only a small circumscribed deposit is found. Fleischhauer (99) has also described a drop method of testing sera which, he states, only disagreed in 2 % of 212 cattle on which it was tested. Sirk (100) described a method which might be of value where no tubes are available. A drop of blood or milk is allowed to dry on a glass slide. At any time up to 3 weeks this drop can be redissolved in saline, and used in an agglutination test. He claims that the results obtained agree well with those of the usual methods, the only difficulties being associated with doubtful reactors to the tube test.

Plum & Russeff (101) have suggested from guinea-pig experiments that a concentrated aqueous filtrate of *Brucella* cultures can be used as an agent for an intradermal test for infection. Grycz *et al.* (102) attempted to correlate with serological tests the results of an allergic test, using a brucellin containing a small amount of "R" material. Only 40 % of the results agreed. Duffau (103) gives details of the methods of isolation of *Brucella* and discusses the intradermal brucellin test.

Bell & Irwin (104) and Irwin & Bell (105) have studied the changes occurring in the blood cells of infected cattle. They found that there was a decrease in the total blood cells (red and white) following infection, but that the relative percentage of polymorphonuclear leucocytes and monocytes increased. The lymphocytes were proportionately decreased. Ber (106) found that in artificially infected guinea-pigs, the number of polymorphonuclear leucocytes rose within the first few hours, and that this was later followed by a rise in the number of lymphocytes. The latter remained high but the former fell to normal after a few days. Priestley (107) examined the phagocytosis of *Br. abortus* by cells from resistant and susceptible animals, using Huddleson's (108) "opsono-cytophagic" technique. He found that it was necessary to use relatively heavy suspensions of *Brucella* (15 × Brown tube 1) if clear results were to be obtained, and that even then in resistant animals agglutination of the bacteria interfered with the test. He was able to correlate phagocytosis with the presence of a somatic antibody in the serum.

Pathogenicity

The natural pathogenicity of *Br. abortus* has been studied by various workers. Fitch *et al.* (109) found that vaginal swabs from infected cows were only positive if discharges were present or the uterine seal broken. They also (110) examined the uteri, post-mortem, of ninety-three non-pregnant animals (positive to the blood test) to ascertain if the bacillus remained for any length of time in this organ. Of these, 51 % yielded Brucella, and the authors therefore consider that this organ may harbour

the organisms for considerable periods (up to 195 days). Wackerbarth (111), on the other hand, examined twenty-two non-pregnant uteri from cows with titres of over 1:500—two with pyometra, three with glandular enlargements and nine with histological abnormalities. In no case, however, was he able to find *Br. abortus* by culture or by guinea-pig inoculation. Fitch *et al.* further (112) examined the saliva of thirty-nine reacting cows. Of these only one was positive and this had recently aborted, the discharges being still present when the sample was taken. The submaxillary and parotid glands were also negative. The same authors artificially infected seven heifers and made periodic blood cultures. They found a Brucella bacteraemia usually running roughly parallel with the agglutination titre. The bacteraemia was demonstrated in all animals up to the time of parturition, but in only two thereafter. Hoppe (113) found that, in the kidneys of aborted foetuses, an interstitial non-purulent nephritis due to a diffuse infiltration by lymphocytes is observable microscopically. The changes correspond to those found in the condition known as "white spotted kidneys". It seems, however, that the latter condition is not due to Brucella infection, since Heinen (114) examined nine calves with "white spotted kidney", and found that all were negative to the agglutination test and that no Brucella could be cultivated from the spots or demonstrated by guinea-pig inoculation. Ariel (115), who examined the organs of sheep foetuses aborted as a result of Brucella infection, states that he found no changes in the kidneys or heart, but that necrotic foci with polymorphonuclear leucocytes were present in the liver and adrenal glands, together with a generalized hypoplasia of the reticulo-endothelial system. He noted particularly that there were acute inflammatory changes in the umbilical cord.

Genital brucellosis of the bull and the possibility of its transmission in coitus has been studied by Jepson & Jørgensen (116). They found the seminal vesicles to be most usually affected, but were unable to transmit the disease by coitus even where a unilateral orchitis was present. The secretion of the seminal vesicles, when in the acute stage, was, however, heavily infected and, though no bulls thus affected were available for transmission experiments, the authors think that in this stage the bulls are probably infective.

Artificial infection

Priestley & McEwen (117) have tested the known toxic (as distinct from infective) effect of intraperitoneal injections of *Br. abortus* in mice. They relate the toxicity of any culture to its virulence and suggest that this may usefully be employed as a virulence test. Priestley (118), in a further endeavour to find a rapid test for the virulence of a culture, examined the necrotic action of Brucella strains injected intradermally in the rabbit. Though some relationship was found, it was not thought sufficiently clear to be of practical use. The reaction is described as starting as a mild inflammation, progressing through intense inflammation to pus formation and sloughing, followed by healing, the whole process taking about ten days. Scorgie (119) attempted to replace guinea-pigs with mice as the test animal for virulence tests, but found by parallel tests that the latter are less susceptible and cannot adequately replace the former. Pullinger (120) has pointed out that some local immunity to *Br. abortus* is produced in guinea-pigs if tuberculin is also injected. This immunity could also be produced by adding *Bact. monocytogenes* to the inoculum. The same result was not obtained if Johnes bacillus, B. C. G. or extracts of *Myco. tuberculosis* were used. It is thought to be due to the local production of macrophages.

Vaccination

There seem to be two lines of work at present in progress, both designed to ascertain if successful vaccination against abortion is possible, using living strains of low or no virulence. In Britain the study seems to be more devoted to the funda-

mental lines of research. McEwen, who has done much work on this subject, has published results (121) which lead him to the important conclusion that the virulence of a strain of low virulence can be enhanced to the full virulence of field strains (as judged by guinea-pig and mouse toxicity tests) by passage through guinea-pigs or by sojourn in the bovine udder. His other conclusions are: (*a*) virulence of a strain to guinea-pigs and mice is directly parallel with the virulence for pregnant cattle, with the important exception that even strains of low guinea-pig virulence may become localized in the uterus and udders of inoculated cows and thus become fully virulent again; (*b*) a strain may be subcultivated many times on laboratory media without being attenuated; (*c*) that it is essential that the vaccinating strain used in cattle should not be capable of localizing in the udder or uterus. McEwen and Priestley (122) tested variants of graded virulence in guinea-pigs, and showed that the resistance conferred was directly proportional to the virulence, though McEwen (123) demonstrated some slight immunity in cattle vaccinated with an avirulent strain. The same author, with Priestley & Paterson (124), has, in an endeavour to find a standard dose for testing purposes, used falling doses of an infective strain instilled in the conjunctival sac. The resulting disease appeared more serious than naturally acquired abortion if the infecting dose was over 1,500,000 organisms. Haring & Traum (125) examined the pathogenic powers of the United States Bureau of Animal Industry strain 19 (a low virulence strain with which most of the American vaccination work is being done). They found that, if inoculated into pregnant animals, it caused abortion, but in non-pregnant animals it was safe to use and did not spread to the non-vaccinated animals. They considered the protective action to last for 2 years and to be effective against any but large doses of virulent bacteria. In a field experiment in a self-contained herd of fifty cows, Haring (126) found that the number of abortions was substantially reduced though a number of the vaccinated cattle became infected. Velu & Zottner (127) concluded from their experiments that a single injection of 4 mg. of living *Brucella* suspended in a non-absorbable excipient produced immunity. A single dose of an attenuated vaccine in an oily excipient was also employed by Dubois (128) on 23,000 animals. The number of abortions was substantially reduced, while an added advantage seems to be that the vaccine may be given during gestation without ill effects. Hardenburg (129) has used a living attenuated vaccine on calves from 3 to 9 months old. A pronounced rise in titre occurred in all, but in 80 % it became negative after 6 months. He compared the results in 143 vaccinated heifers and 73 non-vaccinated. The abortion rate was 2·4 and 6·2 % respectively. Thomsen (130), in Denmark, also used this method with the American strain and found, using 486 calves, that the abortion rate was reduced from 25·1 % in the controls to 3·3 % in the vaccinated group. Demnitz (131) found that growth on media containing bile reduced the virulence so that no organisms were excreted by animals injected by such a culture. Immunity was, however, conferred. Kerr (132) tried the effect of a vaccine made from organisms grown in sterile uterine fluid, incubated for 12 months and allowed to die. The immunity conferred by this vaccine on guinea-pigs and cattle was merely partial. Timoney (92) states that even the protection afforded by live vaccines is only partial and that it breaks down if the animals are exposed to heavy infections, though it may allow a cow to carry her calf longer. He considers, however, that natural infection confers a high degree of immunity to further infection. Beach (133) also found from his observations on a group of twelve cows which had recovered from infection, that re-infection did not cause abortion, neither was the organism recovered from the milk or the uterine fluid. Dead vaccines have also been studied by various workers. In view of the claims made in Germany for the Serobortan-vaccine method, Zeller & Henninger (134) conducted an official test into the value of this method and also of the "AO_3"

vaccine. Neither was of any value. Kress(135) tried a mixture of ten strains of *Br. abortus* killed by formalin. He gave three doses and states that the blood titre then reached 1:100. In 12 weeks it was negative again. His figures for 1633 cows in freshly infected herds show a reduction in the loss of calves from 19·3 to 0·9 %, and in 762 animals in chronically affected herds from 15·9 to 0·6 %. There were, however, no adequate controls.

Control

General articles on the control of contagious abortion in various countries have been published by many authors. In America, Wright(136) states that, during the year 1937, 802,000 herds were under supervision. Approximately 8,200,000 cattle or 15·4 % of the total were under test and the reactor rate had been lowered from 14 to 5 %. A scale of compensation for reactors is allowed by the Federal Government and by some States, while the value of the salvage is also given to the owner. In herds in which the reactor rate is over 15 %, calves from 5 to 7 months old are being vaccinated with live vaccine, about 5000 having so far been treated. De Fosset(137) and the *Report of the Committee on Bang's disease*(138) also give an account of the progress of the eradication scheme. The scheme is run on a nation wide basis and the co-operation of herd owners is stressed. The main difficulties encountered are: (1) lack of co-operation from the farmer, (2) cattle added to the stock, (3) doubtful reactors, (4) interference in the use of vaccines or remedies by non-compliance with instructions, (5) difficulties in testing at regular intervals. However, steady progress is being made. Thirty-five of the States require that cattle for breeding or milking must be free from abortion before entry into the dairy herd, thirty-three demand that all reactors must be marked, sixteen have area testing schemes, and ten have special funds for indemnification. Carroll(139) has summed up the current opinion of some practitioners which seems to be against a strict policy of slaughtering reactors and in favour of calfhood vaccination, though this does not seem to be the opinion of most of the official veterinary officers. The position has been summarized in a report by the Special Committee of the American Veterinary Medical Association(140). Their main recommendations are: (*a*) all reactors to be tagged, (*b*) results of tests to be notified to the authorities, (*c*) control of sales of vaccines, (*d*) frequent (fortnightly) testing with immediate disposal of reactors is often necessary, (*e*) area eradication essential, (*f*) a standard antigen is necessary, (*g*) disinfection of cattle trucks, (*h*) increased educational activity. They state that the Bureau of Animal Industry is engaged on a study of calfhood vaccination in 290 infected herds and 7500 calves have so far been vaccinated. The results are not yet available. Eradication schemes are in progress in 250 counties and 1,750,000 cattle have been tested. The proportion of reactors (including re-tests) is 4 %.

In Britain a discussion by McEwen and other veterinarians has been published(141). McEwen appears to favour vaccination, while others were convinced that eradication was possible if the agglutination test was rigorously applied and proper measures taken. The original should be read by those concerned in control measures, as the "pros" and "cons" of eradication and vaccination are discussed in detail. McEwen(142) has also analysed the cost to the farmers of eradicating the disease, and has shown this to be remarkably small.

In Scandinavia there has been definite progress. In Norway(143), in $3\frac{1}{2}$ years, 2843 out of 2943 infected herds have been cleared. Holth(144) also gives details of the work in Norway, and states that the number of samples examined has now fallen from 74,477 to 32,948, of which 4·3 % were positive, 0·3 % doubtful and 95·4 % negative. These samples were from 5005 herds of which 161 harboured positive reactors. Of 3361 abortions in cattle, only 8·9 % were due to *Br. abortus* (previous

to the scheme 14 % were due to this organism). Holth points out the danger of the cow which, though infected, does not react. Klemola & Knuuttila (145) state that in Finland notification is compulsory and that only about 1 % of the herds are reported to be affected (i.e. about 3000). They consider, however, that this figure is probably lower than the true figure, owing to delay in reporting abortions.

Control measures taken in other countries have been described by Evans (146) in Tanganyika, Gilbert (147) in Palestine, Guillermo *et al.* (148) in Uruguay, Rolle (149) and Roots (150) in the Baltic States (the latter author emphasizes the dangers of using live vaccines), Graub (151) in Switzerland, Simič & Durićié (152) in Yugoslavia, Itabashi *et al.* (153) in Manchuria, Albiston (154) and Hindmarsh (155) in Australia, Alessandrini (156) in Italy (this author states that undulant fever in man reaches its height in April, May and June when the flocks move, and draws attention to the danger of infected manure being used for vegetable growing), Machado (157) in Portugal (who records that the reactor-abortion ratio is lower in swine than in cattle), Ivanoff (158) in Bulgaria (live vaccine is prohibited in this country), Topacio & Acevedo (159) in the Philippines, and the State Scientific Institute at Turkestan (160) (where sheep are particularly affected and the human infection rate reaches 10 %). All control work is on similar lines, viz. blood testing and segregation or slaughter.

In view of the importance attached to control measures in so many countries, two articles by Moore *et al.* (161) and by Martin (162) are of special interest. The authors draw particular attention to the self-limiting nature of the disease and claim that, if left to itself and provided fresh cows are not drafted into the herd, the disease ceases to cause serious loss. Such loss, however, does not appear to take into consideration any wastage due to sterility. Hill (163), writing from the viewpoint of the breeder of cattle, states that in herds from which abortion has been eradicated, the number of services per calf is approximately 1·25, whereas, where abortion is present, the average number of services per calf is five. The respective calf crops are 90 and 50 %. He also points out that vaccinated heifers take two years before they can be bred, leaving this gap to be filled by susceptible animals, and claims that the immunity of vaccinated heifers is not definitely proved. Klemola & Knuuttila (145) state that the average age of the foetus at abortion was 6 months 3 weeks. Cows aborting more than once required more services than others and 41·2 % became sterile. Parschau (164) has brought forward evidence to show that the milk yield is not affected. He studied the milk yields of 5404 cows, of which 1305 had aborted, and was unable to find any difference between the yields of the normal cows and those which had aborted. Marienburg (165), on the other hand, has produced evidence to show that milk is lost for several lactations after abortion, while Klemola & Knuuttila (145) consider the reduction in milk yield in the two lactations following abortion to average 17·8 and 10·4 % respectively. Mazé (166) states that he found that milk from *Brucella*-infected herds retarded lactic fermentation and affected the production of butter and cheese. *In vitro* experiments confirmed this, the inhibitory factor was shown to resist heating at 65° C. for 5 min.

Treatment

The action of the sulphonamide group of drugs on *Br. abortus* infections has inevitably been investigated. Montgomerie (167) reported favourably on its effect in doses of 200 mg. continued for 6 days, followed by 100 mg. for a further 6 days, in four artificially infected guinea-pigs. Welch *et al.* (168) have noted that in affected guinea-pigs treated with sulphanilamide the number of organisms phagocytosed by the leucocytes is increased. This was confirmed by Menefee & Poston (169) in experimental infection of forty guinea-pigs. They also demonstrated the bacteriostatic

powers of the drug *in vitro*. Miller *et al.* (170) attempted to clear infected cows by dosing with this drug. Although a concentration of 20 mg. per 100 ml. of blood was maintained, it had no effect on the disease.

Brucella abortus in other animals

Pallaske (171) examined sixty-three dogs from districts where abortion was unknown and from districts where abortion was endemic. In no case could agglutinins be demonstrated, and artificially infected dogs (*per os*) only showed agglutinins while they were being fed. In horses, Taylor (172) found that of 997 animals 19·6% re-acted to the agglutination test at 1 : 10, 2·1% at 1 : 20 and 1·9% at 1 : 40. There was no correlation between titre and age or sex. Fitch & Dodge (173) divided eighty farms into two groups of forty. In one group all farms had at least one horse with a titre of 1 : 250, while in the other no horse had a titre of over 1 : 25. After eradication had proceeded for some time among the bovines, the first group still had a reactor rate of 3·7% while the second group had only 0·5%. This is presumed by the authors to be due to horses acting as reservoirs for the disease, but there may easily have been other factors since the first group had an original infection rate of 29·2% against 17·1% in the second group. In goats, Doyle (174) has shown that *Br. abortus* is not transmitted naturally from infected to non-infected animals and that, even in artificially infected goats, positive blood titres are difficult to obtain. He was able to infect forty-eight goats artificially (conjunctival method) of which thirty-six aborted after an average period of 54 days. Serial passage through goats did not affect the virulence of the organism for cattle, and Brucella could be isolated from the milk up to 386 days after inoculation. Pavlov (175) studied the disease in poultry and found them difficult to infect. When infected the titre reached its highest point after one month and disappeared in two. An allergic reaction could be obtained up to 3 months. Bosworth (176), Fitch & Bishop (177) and Sandholm (178) emphasize the importance of rats as transmitting agents for the disease. Bosworth found one infected rat out of forty-three from infected farms, and Fitch & Bishop one out of sixty-six similar rats. Both Sandholm and Bosworth fed rats on infected material. Organisms could be recovered from the organs and from the urine (Bosworth) and faeces (Sandholm). Cotton *et al.* (179) were not able to transmit porcine brucellosis from pigs to cattle by natural exposure, but direct implantation of culture into the conjunctivae was successful.

619.2:616–002.5

III. TUBERCULOSIS

Cultural characteristics

A new and simple medium similar but, according to the author, superior, to Herrold's egg-yolk agar, has been described by Jamieson (180), while Bond (181) has studied in considerable detail the various morphological forms of the bacilli in colonies on agar surface cultures. A method of differentiating human from bovine strains has been described by Kliewe & Schuppener (182) who grew both types in sour milk. When tested by injection into guinea-pigs it was found that, while the human type died out rapidly (within 7 days), the bovine type remained infective after 20 days. Wilson & Schwabacher (183) examined suspensions of *Myco. tuberculosis* to ascertain the total number of organisms per mg. of culture and the proportion viable. It was found that a culture 2 or 3 weeks old contained approximately $12–13 \times 10^8$ organisms per mg. and that 55% of the avian, 8·8% of the bovine, and 4·7% of the human type were viable. It was considered, however, that the latter two figures were underestimated.

The resistance of the Mycobacteria to various adverse conditions has been studied by Potter (184) who used a technique which theoretically removed all oxygen and water from the bacilli, i.e. they were completely desiccated. They survived this treatment for from 12 to 24 months. The resistance shown to the effects of heat and of acidity was examined by Corper & Cohn (185), who state that the type of medium has some effect on the ability of the organisms to withstand the effects of these agents. Egg medium was found to be favourable to survival, and the avian type was the most resistant. Bongert (186) considers, on the basis of experiments on the feeding of calves with holder pasteurized milk, that heating to 60–63° C. for 30 min. can be resisted by the bacilli which, Bongert claims, can still infect calves. This conclusion is at variance with that of most other recent investigations. Corper *et al.* (187) investigated the efficiency of irradiation by short-wave wireless waves, but could not detect any lethal effect either *in vitro* or *in vivo*.

Arloing *et al.* (188) studied the dissociation of R and S colonies of human and bovine *Myco. tuberculosis* which had been grown for 25–41 years on culture media. The bovine strains dissociated less readily than the human, and when passaged through rabbits yielded fewer R colonies than the human strains. The effect of the culture medium on the virulence, i.e. the S-R variation, has also been studied by Smithburn (189), who stresses the influence of *p*H on the dissociation of the tubercle bacillus. A *p*H of 6·8 gives S colonies of full virulence, whereas if the *p*H is 6·0 the R type predominates, with consequent loss of virulence. The S forms are not considered by Chiti (190) to behave sufficiently characteristically, at least in liquid media, for any differentiation between mammalian and avian strains to be possible by this means.

Synthetic media are being much studied both from the point of view of the metabolism of the organism, i.e. the essential elements for its growth, and from the necessity of producing tuberculin as free as possible from protein contamination. In this connexion may be mentioned the protein-free medium described by Lockemann (191), in which the only organic compounds are glycerin and asparagine. Sher & Sweany (192) have demonstrated the stimulating effect of the ions of iron salts on the growth of *Myco. tuberculosis* on pure synthetic media, especially if copper, manganese, or pyro-antimonate ions are also added. They also observed that if an oxidation inhibitor such as diphenylamine were added, growth was completely prevented even at dilutions of 1 : 18,000, and greatly retarded at 1 : 50,000. Other substances of the same type gave similar results, as did $CuSO_4$. The reaction of the various types of tubercle bacilli to synthetic media of different kinds has also been studied by Nikolić (193), who considers that the variations are sufficient to distinguish between the types, though Groh (194) was not able to separate mixed cultures of human and bovine tubercle bacilli by cultivation on synthetic media. These media have also been used for the study of the specific polysaccharides of bovine tubercle bacilli by Menzel & Heidelberger (195). They showed that this type was more complex than the human, and that the serological type of any strain was related to its pentose fraction. McCarter *et al.* (196) studied the precipitin reactions of the acid-fast group and concluded (using one human, one bovine, five avian, one Johne's and two saprophytic strains) that all were distinct, except that the avian could not be distinguished among themselves and (more remarkable) that it was not possible to differentiate between the avian type and the Johne strain. This close relationship may explain the sensitivity to avian tuberculosis of cattle suffering from Johne's disease. Chiti (197) has also studied the avian tubercle bacilli serologically, and has shown that it differs antigenically from the mammalian types and that, in contradiction to the conclusions of the above authors, it does not form one homogeneous group. Suzuki (198) found that ninety-eight human strains could be divided into one main and two small groups. The three bovine strains he examined were serologically identical.

Production of tuberculin

The value of synthetic media for the production of tuberculin has been generally admitted. Boquet(199) has described the methods of preparation used at the Pasteur Institute. As the protein (i.e. the tuberculin) content could be estimated in such media, it was possible to check the production of tuberculin more accurately. It was found that the bovine strain produced its maximum strength at the sixth week, as against the twelfth week for the human strain. It might be noted that Boquet used the phosphotungstic-sulphuric acid method of precipitation, as against the ammonia sulphate method used by English workers (see Buxton & Glover(200)).

As to the best medium to be used for the preparation of tuberculin, Celoria(201) considers, after a series of comparisons of growth and yield, that Sauton's medium has advantages over most others. Doig *et al.*(202) have described the preparation and testing of a protein fraction (P.P.D.) from tuberculin prepared from synthetic media using Seibert's method. The bacillus is grown on synthetic protein-free media and the product steamed and evaporated. Most of the impurities are removed by ultra-filtration, and the active protein fraction precipitated out with trichloracetic acid. The precipitate is washed in this reagent and the acid is then removed with ether which automatically dehydrates the protein. The dry powder so produced is stated to be of constant potency, stable up to 3 years and to be non-sensitizing. The reactions given by it when redissolved in saline are similar to those of synthetic medium Old Tuberculins. P.P.D. has been employed in testing cattle by Montgomerie & Thomson(203), 352 free and 74 positive cows being tested. It was found that, if used at one-third International Standard potency, it detected animals of low sensitivity and gave few pseudo-positives.

The general question of the potency of tuberculin has been much discussed in Britain. It is known that if the potency is too high, many pseudo-positive reactions occur; if too low, many positive animals are missed. Montgomerie & Thomson(204) have tested two tuberculins, prepared similarly but from different strains by different laboratories, on 579 cattle, and found considerable disagreement in the positive cases, the more potent tuberculin giving 14·2% more positives. The results agree with those obtained with the U.S.A. Department of Agriculture tuberculin (which is of high potency), and the authors consider therefore that they were correct. Unfortunately few post-mortem examinations could be made, and though these showed that a number were true positive infections, there were also a number of false positives. The authors consider the differences in reaction to be due to the differences in potency, but think that the advantages of the higher potency, i.e. no false negatives, outweigh the disadvantages, i.e. false positives. Blount(205) has also drawn attention to the differences between two tuberculins, one of which gave twelve positive reactions and the other six. No post-mortems were, however, made. Just what potency is the best for practical use is not yet clear.

The value of tuberculin as an agent for the diagnosis of tuberculosis has been discussed by authorities in several countries(206). Mohler noted the difficulties in the U.S.A. as a result of non-specific reactions due to "skin tuberculosis", and stated that such reactors are isolated and re-tested, Plum drew attention to the occurrence of avian tuberculosis in Denmark, while Zeller urged an international standard for tuberculin. Glover reviewed recent developments and emphasized that, to be successful, tuberculin must be denatured, must be produced from suitable strains, must be of correct potency, and must be standardized by testing on both positive and negative cattle. He also pointed out that, when re-tested, cattle should not be injected within 6 in. of the first site.

Pathogenicity

Considerable interest has been displayed in the pathogenesis of tuberculosis in calves. Strube (207) examined sixty-eight gravid uteri from tuberculous cows, and found one case of affection of the foetus. Plum (208) estimates from several hundred examinations of aborted placentae that approximately 1 % of abortions in Denmark are due to tuberculosis. An interesting fact is that when he injected four pregnant cows intravenously with avian bacilli, three aborted after about 100 days. Post-mortem inspection revealed infection only in the uterus. He considers that genital tuberculosis due to the avian type of infection is increasing in Denmark. In connexion with uterine tuberculosis the experience of Machens (209) may be noted. Of 151 cows examined as a result of tuberculosis being present in their calves on slaughter (under 3 weeks old), no less than fifty-one had uterine tuberculosis. Rautmann (210) issued a questionnaire to abattoirs and studied the distributions of lesions in 174 cases of infection in calves under 14 days old. In 319 of the animals the disease was generalized, and in some cases the primary lesion was in the mesenteric lymph nodes. These latter are considered to represent infection *in utero* from the amniotic fluid. The remaining cases are presumed to be infected via the umbilical vein from the placenta. Hermansson (211), who examined 313 cases of congenital tuberculosis, states that the portal lymph nodes were affected in 99 % of cases, the kidneys and renal lymph nodes in 14 % and the muscles in 12 %. This evidence presumably indicates infection via the umbilical vein in 99 % of the cases. Schultz (212), who made a careful examination of the distribution and character of the lesions in 107 tuberculous calves up to 16 weeks old, also concluded that congenital tuberculosis was umbilical in origin and that ingestion tuberculosis only occurred in calves more than 4 weeks old. The majority of cases (54) were of the respiratory type. Whether these were true respiratory infections is doubtful in view of the results of Wilkie *et al.* (213), who found that the primary lesion cannot always be found in the mesenteric glands even in cases of ingestion tuberculosis. These workers fed thirty-six young calves with tuberculous milk, and on autopsy twenty-three showed tuberculous lesions in the lungs, mediastinal and bronchial glands. No primary lesions could be detected in the mesenteric glands. On the other hand, Schumann & Fritzsche (214) gave four calves tuberculous milk varying from lightly to heavily infected, and found tonsillar and intestinal tuberculosis in all. These calves were, however, examined at a considerably earlier stage than those of Wilkie *et al.* Hemmert-Halswick (215) fed sixteen pigs with 3 mg. of bovine bacilli; all showed generalized tuberculosis, including the mesenteric glands. Histological examination revealed a primary lesion in the small intestine (usually the ileum) in thirteen of the animals, while primary lesions in the pharyngeal glands were observed in all except those in which the dose had been given in a gelatine capsule.

The possibility of incomplete primary complexes of tuberculosis in the intestinal mucosa or the lymph nodes appears to have been overlooked by many workers who have previously reported apparent respiratory infection. Their importance is, however, now recognized. A discussion of the state of knowledge of the pathogenesis of calf tuberculosis has recently been published by Hildenbrand (216). This author considers that, though calcification of the primary lesion can be detected microscopically after about 3 weeks, no macroscopic changes occur before the fiftieth day after infection. Kiedrowski (217) also considers that primary intestinal tuberculosis is more frequent than is usually reported. In ten calves, between 6 and 12 weeks old, he found six with complete primary complexes and four in which the mesenteric glands alone were infected, i.e. the intestinal lesions had healed. A case of tuberculosis in a calf has also been fully described by Fölger (218).

Lange (219) has shown in experiments with sheep (using bovine bacilli) that, where the primary complex is quiescent, superinfection does not cause activity. Feldman & Baggenstoss (220) could only demonstrate, by guinea-pig inoculation, living bacilli in the healed primary lesions of one case out of sixty-eight. It is, therefore, not definitely proved that the healed primary foci do not contain living bacilli. Jansen (221) has shown that Koch's phenomenon occurs even when the re-infecting bacteria are taken from a lesion already present in the body. Thus it is doubtful if re-infection can occur without some factor other than the bacteria being involved. Aubertin *et al.* (222) injected a strain of low virulence to form a primary focus and then superimposed an infection of fully virulent bovine bacilli. The osteoarthritic lesions observed were found to be due to the original strain of low virulence, presumably due to a flare up on the primary lesions, while the lesions produced by the virulent strain were not of such an extent and nature as this strain produced in animals not previously infected. Smithburn (223) has shown that, in guinea-pigs experimentally affected with tuberculosis, young animals, as judged by the survival time after infection with a lethal dose, are more resistant than old. Kon & Maddock (224) studied resistance to tuberculosis in relation to the vitamin A content of the food. No evidence was obtained that lack of this vitamin increased susceptibility to the disease.

Though not strictly referring to resistance, the results of Schulte (225) are interesting. In a study of eighty-two cases of chronic tuberculosis, he found tuberculosis in the adrenal and thyroid glands but not in the hypophysis, the pineal, the thymus or the parathyroid glands. Joyner & Sabin (226) have produced evidence in guinea-pigs to show that the area of spread of dyes injected intradermally is less in allergic guinea-pigs than in normal, and hence there appears to be some change in the permeability of the skin in cases of tuberculosis. Howe (227) has shown that, at least in experimental tuberculosis of the dog, a sporadic bacillaemia occurs, but Benedetto (228) failed to find any evidence of bacillaemia in sixty-two actually affected cattle using Lowenstein's technique. Thijn (229) found certain changes in the blood of affected cattle characterized by a slight anaemia and leucopaenia, together with a tendency to monocytosis and the presence of typical neutrophils. Though not significant, the changes are considered indicative of tuberculosis.

The occurrence of infection with the bovine type of bacillus in animals other than cattle has been studied by several workers. Plum and Slynborg (230) note that in a district with a high incidence of tuberculosis in cattle, no less than 28% of the swine slaughtered were affected with tuberculosis. Craig & Davies (231) describe a case of extensive infection in a sheep. Bacilli were found microscopically, and animal inoculation revealed infection with the bovine type. Another case of bovine tuberculosis in the same animal has been described in detail by Stubbs and Live (232). Wells (233) considers that human and bovine strains of tubercle bacilli can be distinguished by the great difference in their infectivity for voles, which were found to succumb to 0·00001 mg. of bovine bacilli, whereas it required 0·1 mg. of the human type to cause death. Griffith (234) showed that the vole, though susceptible to bovine, human, avian, and vole strains of tubercle bacilli, yet react differently to each. His results can be summarized as follows:

Bovine type: whether of high or low virulence for rabbits; a rapid progressive tuberculosis with glandular caseation.

Human type: usually a local microscopic lesion with generalized presence of organisms throughout the body without microscopic lesions.

Avian type: as for human but less tendency to form local microscopic glandular lesions.

Vole type: generalized tuberculosis with masses of bacilli forming large necrotic or caseous areas in the areolar tissues. The disease is more chronic than that caused by the bovine type.

There is thus considerable evidence that the vole is a very useful animal to use in differentiating between bovine bacilli of low virulence to rabbits and the human type. Griffith & Pagel (235) also tested these four types on the Golden Hamster and found that this animal, while reacting more to the bovine than the human strains, was of no value for differentiating definitely between the two. The avian strain produced the usual type of avian infection in mammals (generalized but no macroscopic lesions), while the vole strain differed from the other mammalian types in that, though giving anatomical tuberculosis, there was no caseation of the lesions.

Saenz & Canetti (236) have injected heat-killed bovine and human types suspended in vaseline into rabbits, and found that the lung lesions were much more severe with the former type and that they differed only quantitatively from the lesions produced by the injection of living bacteria. They argue that the lesions are presumably caused by the chemical compositions of the bacteria and not by their living processes. Negre & Bretey (237) have also demonstrated the difference between the pathogenic effects of killed cultures when injected in saline or in liquid paraffin. In the former local lesions only are produced, in the latter generalized lesions, even including miliary tuberculosis, are observed. The lesions are epitheloid nodules surrounded by lymphocytes and fibrosis containing the bacilli and the excipient in the centre.

Bovine infection in man

The question of bovine infection in man has also received attention. In Holland, Boer (238) reported on six cases of pulmonary tuberculosis due to this type out of 206 investigated. In Germany, Goeters (239), Heymer (240) and Lange (241) have reported on a total of 291 examinations, of which twenty were found to be due to the bovine type. Oesterreich (242) examined 503 cases of tuberculosis in several areas, and found 20% had their primary complex in the mesentery, i.e. were presumably ingestion tuberculosis. He considered that this pointed to infection from milk and hence to bovine type infection, but he carried out no bacterial tests to confirm the type present. Lange estimates from his work that 25% of all cases in country dwellers are due to the bovine type. Petragnani (243) states that in Italy bovine tuberculosis in man is rare, and that only ten cases have been reported since 1918, while Rosa (244) found one case in 147 of pulmonary tuberculosis. The U.S.A. is also relatively free from bovine tuberculosis in man. White (245) reports on nine cases out of 227 non-pulmonary infections. He notes that all milk supplied to towns is pasteurized, and that country dwellers are not exposed to much risk owing to the rarity of tuberculosis in cattle. Pikkarainen (246) states that he was unable to find any bovine bacilli in a series of 140 human cases examined by him in Finland, in which country only 0·1% of the cattle are infected. Morgan (247) reviews the records of cases in Scotland, and quotes the results of various workers who find that the incidence of the bovine type varies from 4·59% in pulmonary tuberculosis to 80·7% in the abdominal type. In France, Rocher & Laporte (248) report finding the bovine type in two cases out of a series of eighty-nine, and Saenz (249) in twenty-six out of 1199 cases of synovitis of the hands in butchers due to infection by the bovine type of bacillus following cuts.

Immunization

The questions of the allergic state to tuberculosis and of vaccination have been studied by several workers. Of vaccines the best known is the avirulent, B.C.G. type.

Weissfeiler *et al.* (250) compared B.C.G. with ten other vaccines, using rabbits and guinea-pigs as their experimental animals. B.C.G. was found to be the most satisfactory, but the effect varied with the age of the culture, one, 10–15 days old, being

best. They also examined the power of saprophytic acid-fast organisms to protect against further infections with virulent tubercle bacilli. They found that not only was no protection conferred, but that the opposite effect was produced, the lesions being more severe than in the controls. It was noticed that, while B.C.G. had no effect on the lymphatic system, most other vaccines produced an hypertrophy of this system. Immunity due to B.C.G. was found to last, under the experimental conditions, for about 4 months. Opie & Freund (251) compared B.C.G. with a heat-killed vaccine, and stated that the latter was but little inferior to the former, as judged by the results following lethal doses of bacteria. Le Chiuton *et al.* (252) have found that they were unable to increase the pathogenicity of B.C.G. by the action of acetone, methyl alcohol, or tuberculin, though these agents would increase the virulence of strains of tubercle bacilli of low virulence.

Griffith & Glover (253) examined five vaccines using guinea-pigs. Four were dead virulent bacilli (killed by heat at 60° and 120° C. and by formalin and glycerin respectively), and the fifth was B.C.G. There was some evidence (slightly in favour of B.C.G.) of raised resistance of no high order to subsequent infection with tuberculosis in most of the vaccinated animals, but in only 50% were they able to reduce the extent and severity of the generalized tuberculosis. Gräub & Zschokke (254) also examined the effects of four vaccines (antiphymatol, B.C.G., Friedmanns (turtle bacillus) and a slightly virulent living vaccine). The results showed no protection with the antiphymatol, delayed death with B.C.G. and Friedmanns, and death from the vaccination with the virulent strain.

Dessy (255) has published results using the Belfanti-Dessy vaccine (a live vaccine in saponin). The vaccine is injected at the base of the tail and gives a local nodule without generalization. About 250 cattle were vaccinated over a period of 5 years and only two contracted tuberculosis, although all were exposed to infection. Two cows injected intravenously with virulent bacilli 8 months after vaccination did not contract tuberculosis, while two others injected subcutaneously only showed a local lesion. Controls contracted generalized tuberculosis. Unfortunately only a few details are given, and it is difficult to judge the real value of these observations. Buxton & Glover (256) tested the effect of the injection subcutaneously into calves of B.C.G. vaccine suspended in oil. Their conclusions were: the subcutaneous inoculation of three calves with 5 mg. B.C.G. incorporated in gum acacia and olive-oil failed to induce an appreciable immunity to the oral administration of cultures of virulent tubercle bacilli 6 months later. When the dose of B.C.G. was increased to 50 mg., a moderate protection was obtained in three calves out of four. A reinforcing inoculation of B.C.G., given under the same conditions 12 months after a primary dose, induced a partial protection to the same infecting dose administered 6 months after the second injection of the vaccine. There were marked individual differences, however, since three calves out of six were completely protected, while in the remainder the lesions were almost as severe as in the controls. There was thus no clear indication of any immunity.

That dead vaccines can at least produce an allergic state has been shown by Hensel (257), who points out that a far better and more lasting response is obtained if the vaccines incorporate lanoline, vaseline or paraffin. Coulaud (258) used dead vaccines in olive-oil, rancid olive-oil and castor-oil and compared the results with the same vaccines suspended in saline, paraffin or lanoline. He states that, when injected subcutaneously, the oil vaccines gave more protection than the saline, but less than the paraffin or lanoline.

A study of the actual mechanism of tuberculous immunity in guinea-pigs and rabbits has been published by Laurie (259). He deals mainly with local immunity at the site of inoculation and the effect of the tissue exudates on the bacteria in reinfected

animals. His main conclusions are that local immunity is due (in the guinea-pig) to thrombosis of the lymphatics and the formation of a film or network, together with a local increase in the antibody content of the tissue fluids.

Diagnosis

The presence of tuberculosis, or at least of an allergic state to the products of this organism, has been widely studied by the use of various tuberculins. Tuberculins varying from those normally employed have been described by Arena (260) who uses an aqueous extract of tubercle bacilli and claims good results. Gottschall & Bunny (261) describe a special diluent which is said to keep the tuberculin more stable. Purified protein derivative (the latest form of pure tuberculin) has been compared favourably with Old Tuberculin by Seidman (262). A general review of the subject has been published by Buxton & Glover (200) in a special report of the Agricultural Research Council. This particularly applies to Great Britain and summarizes the results of 15 years' work on tuberculins. The authors emphasize the great improvement brought about by the introduction of synthetic media tuberculins in the reduction of doubtful and pseudo-positive reactions, though they also point out that non-specific substances, when injected near the site of a tuberculous reaction, may give pseudo-positive reactions in sensitive animals. This phenomenon is, however, transitory and the area around the injection then becomes less sensitive than normal. This phase lasts about 6 weeks. One point of practical interest is that repeated injections of unheated tuberculins were found to cause an allergic state to be set up with consequent reaction to the tuberculin test. If heated tuberculins were used no such reactions occurred and these are, therefore, to be recommended for use in field control. Reactions to mammalian tuberculins were also obtained by infection with avian tubercle bacilli and *Myco. Johne. Myco. phlei, Corynebacterium pseudotuberculosis, Actinomyces bovis* and *Actinobacillus* did not sensitize animals to tuberculin. If avian tubercle bacilli were injected, the response to mammalian tuberculin was transient, whereas that to avian tuberculin was sustained. Bonnet *et al.* (263) examined the character and duration of the reaction to mammalian tuberculins in fowls, guinea-pigs, and rabbits infected with avian tuberculin. It was found that, as compared with the reaction to avian tuberculins, the reaction was delayed, disappeared more rapidly and varied more in intensity. The question of infection of bovines by avian tuberculosis has been much studied recently, since, as eradication of tuberculosis from herds has proceeded, more and more cases of reactions to tuberculin in supposedly clear herds have been traced to this type of infection. Particular attention has been paid to this problem by Plum (264) in Denmark, who has recently published several articles summarizing his many years' work. His conclusions (1937) are broadly as follows:

1. Repeated intradermal testing at short intervals tended to reduce the sensitivity to tuberculin.

2. Massive doses of tuberculin, either intravenously or subcutaneously (20 ml. on each of three successive days), practically destroyed the sensitivity to tuberculin. This is in contradiction to the findings of Buxton & Glover (200).

3. Avian tuberculosis in the bovine could not be differentiated from true bovine tuberculosis with certainty by any of the following methods: (*a*) Re-testing with avian tuberculin as well as bovine tuberculin; if the former gives the greater reaction it is probable that the animal is not affected with bovine tuberculosis, but it is not certain whether it is affected with avian tuberculosis or with "skin lesions" or Johne's disease. (The differentiation between the reactions to the tuberculins is in any case often difficult to make.) (*b*) Application of the subcutaneous or ophthalmic tests

instead of the intradermal test. (*c*) The use of falling doses of avian and bovine tuberculins.

4. Avian tuberculosis artificially induced in animals already infected with bovine tuberculosis was not found to interfere with the diagnosis of bovine tuberculosis (the normal reactions of an animal suffering from bovine tuberculosis to bovine and avian tuberculin is as 4 : 1 in intensities, which, where super-added avian tuberculosis occurs, is brought down to 2 : 1).

5. In a series of post-mortems in animals reacting to the tuberculin test, the avian bacillus was found in thirty-five out of forty-eight mesenteric lymph nodes. The other lymph nodes were less often affected.

In a more recent publication, Plum(265) has summarized his views on the differentiation of bovine, human and avian tuberculosis in cattle. He considers that where the mammalian tuberculin gives the larger response, the infection is due to bovine or human tuberculosis. Differentiation of these two types is only possible by the history of the herd and attendants. If avian tuberculins give a bigger reaction, then the cause may be either avian tuberculosis or Johne's disease. The only method of differentiation is the clinical history. The reactions were found, according to a further article(266) by this author, to be greatest in animals recently affected, i.e. up to 1 year old, or, if older, showing few lesions. He examined post-mortem 2079 cattle giving positive reactions to Old Tuberculin, and found that the test, so far as positives were concerned, was 96·7% correct. Plum's findings have been applied by Stenius(267) in Finland apparently with success for the differentiation of the three types of infection *in vivo*.

Feldman(268) has recently reviewed the literature on avian infection in bovines, and has given a summary of recent cases totalling ninety-six. He sums up the present position as follows: (i) avian infection causes either no lesions or only small local and non-progressive ones, usually in the alimentary lymph nodes; (ii) lymph nodes showing no macroscopic changes may yield avian bacilli on culture; (iii) cattle affected with avian tuberculosis react to avian and mammalian tuberculins but usually the sensitivity is transitory. This author has also published a book on the subject of avian tuberculosis(269). That the avian bacillus can on occasion cause lesions in the bovine has been shown by Timoney(270), who recorded a case of extensive tuberculosis involving the udder in which only avian bacilli were recovered from the milk. Unfortunately no cultures could be made from the other affected parts. The culture obtained from the milk was used to inject quarters of two non-tuberculous cows. They both reacted to the tuberculin test and, though bacilli could still be recovered from one cow at the time of slaughter, there was no macroscopic evidence of tuberculosis of the udder. Griffith(271) has reported that avian bacilli were recovered during a period of nine years from the udder of a goat (artificially infected) and, though the affected quarter atrophied, no sign of tuberculosis—even microscopically—was visible at post-mortem two years later. It is interesting to note that just before the last positive cultures were made the organism had changed from the S to the R form.

Russeff(272) points out the necessity of eradicating avian and porcine tuberculosis if a bovine eradication scheme is to be a success. Nielsen & Plum(273) have shown that the transmission of bovine tuberculosis in cattle from infected humans can, in some cases, reach quite large proportions. In seventeen herds, comprising 632 cattle, they traced outbreaks of tuberculosis to human contacts. In all, 384 animals became infected, i.e. over 50%.

Tuberculosis in twelve sheep and ten goats in Uganda is described by Carmichael(274), in a goat in Ireland by Begley(275) and in England by McKay(276), and in a roe deer in Germany by Schmidt(277).

Another difficulty in the tuberculin testing of animals (not including practical

details of technique and interpretation for which those interested may refer to Tobler(278), Smythe(279), Blanchard(280), Hancock(281), Pröscholdt(282), Rolle(283) and Hungerford(284)) is the occurrence of delayed positive reactions as mentioned by Cabot(285). He found that of 16,119 cattle in 285 herds tested for the first time, no less than 935 which had been negative or doubtful at the 72nd hour (to the double intradermal test) became positive by the 120th hour. He suggests that in herds tested for the first time, the reading at the 72nd hour be omitted and one at the 96th hour substituted.

The presence of so-called "skin tuberculosis", first described in America, is also a matter of difficulty. Cows so affected react to the tuberculin test and should presumably be removed from the herd. This is not, however, the view of Andersson(286), who considers that such animals should be retained as, in his experience, the infection occurs as an epizootic and removal of the affected animals does not prevent the spread. Recovery usually occurs fairly rapidly. Krantz(287) also describes this condition and reviews the literature up to 1938. He was able to demonstrate acid-fast bacilli in the lesions, but all attempts to obtain cultures or to infect guinea-pigs failed, as did those of Hole & Hulse(288) who have reported on the occurrence of the disease in Great Britain.

Methods other than tuberculin testing of cows are also employed to diagnose tuberculosis, mainly, however, to demonstrate the presence of tubercle bacilli in the milk. One (the so-called Triboulet's test) which consists of examining faeces for specific breakdown proteins, is, however, used for the detection of intestinal tuberculosis. Schmidt & Messing(289) applied the test to 124 animals; sixteen were positive, of which eleven showed no evidence of tuberculosis on post-mortem, and 108 were negative, of which twenty-one were infected. In these workers' hands the test was thus of no value. Ritter & Nothdorff(290) examined 20,000 cow or quarter samples of milk by a shortened complement fixation test. All the samples would normally have been examined microscopically or by guinea-pig inoculation. They found, however, that this test, though it gave many false positives, gave few false negatives (only twelve out of 14,011). Thus they would have been able to discard this number of samples in a routine test, as the number of false negatives is much less than by the other tests. The false positives (false in so far as though the cow might have been tuberculous, she was not excreting the bacilli in the milk as judged by the microscopical or biological tests) must, of course, be put through the usual routine; nevertheless considerable work is saved.

The diagnosis of udder tuberculosis by the examination of milk samples for the bacillus is widespread. Thomas(291) has recently published an improved method of preparing the samples for examination. An ammonia-alcohol solution is mixed with the milk, the mixture left for 2 hr. and then centrifuged at 5000 r.p.m. for 1 hr. It is claimed that this separates the fats and converts the casein into soluble ammonium caseinate. A minimum deposit is thus obtained, and can be examined microscopically. If cultural or biological tests are to be carried out, the deposit must first be neutralized. This method also has the advantage of destroying most of the contaminating bacteria. That this may have a useful application in practice (if the results obtained are confirmed by other workers) is shown by the difficulties encountered by Legrand *et al.*(292), who found that 66% of their cultures—made after treatment with 15% H_2SO_4 for 30 min.—were contaminated with sporulating aerobes, and though this treatment reduced considerably their losses in guinea-pigs through intercurrent infection, it also reduced by 50% their positive results by the biological test. They consider that the main cause of these difficulties is the heavy deposits of coagulated casein which are avoided by Thomas's technique. Pallaske(293) examined post-mortem forty-three udders with tuberculosis, and found that twenty-seven were not

suspected before examination. In most cases no enlargement of the supramammary gland was palpable. He emphasizes that tuberculosis of the udder is "open" from the start of the infection.

Two useful articles showing the value of direct cultural examinations for the diagnosis of tuberculosis when modern methods are used are those of Murphy & Duerschner (294) and Guggenheim & Finkelstein (295). The former authors treated 171 microscopically negative samples with NaOH and, after neutralization, sowed them on Petragnani's medium and injected guinea-pigs. Thirty-one were culturally positive and only twenty-six biologically positive. It was also noted that the number of contaminated cultures was far less than the number of guinea-pigs dying during the observation period. The latter authors also obtained positive cultures with negative guinea-pig findings, and suggest that routine examination should be (i) microscopical, (ii) all negatives cultured, (iii) all negatives to be injected into guinea-pigs. An annotation on the subject of cultural methods for the diagnosis of tuberculosis has been published by Glover (296), who suggests that they should be more widely employed than at present.

Control

The whole question of tuberculosis eradication and control has recently been discussed by authoritative workers at the 13th International Veterinary Congress (297). There is general agreement that the intradermal tuberculin test with synthetic media tuberculin should be used as the diagnostic agent, followed by segregation or disposal of reactors. It will be simplest to deal with the results under the different countries.

1. *United States*

Wight (298) states that in the year to 30 June 1937, 13,750,000 cattle were tuberculin tested, with 0·7% reactors. There were 272,000 accredited herds and ninety-three more counties qualified as areas of less than 0·5% infection. Faulder (299), dealing with the eradication scheme in New York State, states that the incidence of tuberculosis has been reduced from 40% in 1919 to under 0·5% in 1937. The cattle population of this state is approximately 2,000,000 in 153,000 herds. During the full period 965,000 reactors were destroyed, and $57,000,000 compensation paid. It is proposed in future to test the major portion of the cattle annually.

The Special Committee on Tuberculosis (300), reporting in 1939, states that all the States in the Union except California were modified tuberculosis-free areas. Of the 13,000,000 cattle tested in the year ending May 1938, 0·65 were reactors. The importance of avian tuberculosis in provoking reactions is emphasized. It is interesting to note (301) that every herd in the United States has now been tuberculin tested.

2. *Britain*

The number of tuberculosis-free herds in December 1939 was 13,835. Rowlands & Montgomerie (302) have published the results obtained in an upland breeding area in Wales. At the initial test 5% of 470 animals on thirty farms reacted. After 2 years the percentage was 0·5% of 572 animals on thirty-nine farms. The value of repeated tuberculin tests at short intervals in eradicating the disease from herds where difficulty had been experienced in freeing the herd with six-monthly tests has been emphasized by Kelland *et al.* (303). From 1917 to 1930 a herd of 250 head had been tested at 6-monthly periods but infection persisted. The authors substituted 60-day periods for the 6-month periods, and increased the care in disinfection, etc. Within 3 years the herd was free. A number of anomalous results were obtained and are attributed to avian and "skin tuberculosis". Allan (304) states that in "flying herds" the incidence is 42%, in mixed herds 30%, and in breeding herds 3·6%.

3. *Scandinavia*

In Finland (Stenius (267,305), Pikkarainen (246)) emphasis is laid on the presence of the human type of infection in bovines. It was found that, although in abattoirs the percentage of infection was only 0·02%, the percentage of reactors was never less than 4. Further work showed that the difference was due to human type infection, which gave no lesion. In eradication work, therefore, the tuberculin test is used to differentiate between the two types of infection. Those animals considered to be infected with the human type are left with the clear animals, and the source of infection is traced by the medical authorities and removed. Hermansson (211), in Sweden, emphasizes the importance attached to genital tuberculosis in that country. The dams of all calves found affected at slaughter are traced, and the vaginal discharges tested for tuberculosis. If found, the animal is slaughtered. In Denmark, Nielsen (306,307) gives details of the results obtained in some districts where a voluntary co-operative scheme is in force. The State contributes by various means, including a guaranteed price for animals slaughtered for tuberculosis, and it appears that the number of tuberculosis-free herds is steadily increasing.

4. *Germany*

Weisstanner (308) tested 537 animals with various tuberculins by the subcutaneous, intradermal and conjunctival methods. Of 143 non-reactors to the subcutaneous test he found no less than sixty-eight positive to tuberculosis on post-mortem. He considers, in common with most workers, that the double intradermal test is the best. Pröscholdt (309) also advocates the intradermal test, and states that of 30,000 animals examined, 50% of the cows, 40% of the bulls, 30% of the young animals and 14% of the calves reacted.

5. *Holland*

Both Venema (310) and Beijers (311) are agreed that the position in Holland is unsatisfactory. Both argue for more co-operation between the veterinary and medical professions, but they differ fundamentally in their suggestions for the better control of the disease. Venema considers that the scheme must be compulsory with penal clauses for non-compliance. Beijers, on the other hand, believes that successful results will only be obtained by the willing co-operation of the farmer. He thinks that some of the results so far obtained are fairly satisfactory, and instances the district of Utrecht, where in 5 years the proportion of reactors has been reduced from 32·6 to 20·9%. Doeve (312), in the Dutch East Indies, is strongly against the destruction of reactors on the ground that the double intradermal test is not free from error and gives false positive results. He considers that reactors should only be slaughtered if clinical or bacteriological signs are present.

6. *Other countries*

In Yugoslavia tuberculosis is rare. In the last 10-year period it was only observed in 0·51% of 200,000 cattle slaughtered at the abattoirs (Debelić (313)). In Portugal energetic steps are being taken. According to Lucas (314) and Anon (315), clear areas are being established. A standard tuberculin, injected into the caudal fold (one injection), is used by official veterinary officers. All animals are branded and movement is controlled. Ivanoff (316) states that in Bulgaria, of 35,000 cattle tuberculin tested in 1937, 2·13% reacted. The regulations in this country contain a provision for the compulsory tuberculin testing of all cows and buffaloes giving milk for human consumption. From Rolle (317) it appears that in the smaller Baltic States, control

measures are not far advanced. In Australia, Holmes (318) has stated that about 8% of the dairy cattle are infected with tuberculosis. Little appears to have been done to control the disease, but some steps are now being taken. A full discussion of the position in Australia appeared in the *Australian Veterinary Journal* (319). Ferauge (320) states that in Belgium 25% of the cattle are infected, whereas in South Luxemburg only 10% are tuberculous. He reviews the laws on the subject and emphasizes the necessity for co-operation by the farmers.

P. S. WATTS

THE HANNAH DAIRY RESEARCH INSTITUTE,
KIRKHILL, AYR, SCOTLAND

REFERENCES

(1) UDALL, D. H. (1937). *Cornell Vet.* **27**, 395.
(2) MINETT, F. C. (1938). *J. Fmrs' Cl., Lond.*, Part 5, p. 75.
(3) STABLEFORTH, A. W. (1938). *Agric. Progr.* **15**, 8.
(4) (1938). *Rep. First Imp. Vet. Conf., Lond.*, p. 62.
(5) STABLEFORTH, A. W. (1939). *Vet. Rec.* **51**, 469.
(6) MINETT, F. C. (1939). *J. comp. Path.* **52**, 266.
(7) (1940). *J. Coun. sci. industr. Res. Aust.* **13**, 19.
(8) KLIMMER, M. & HAUPT, H. (1938). *Berl. tierärztl. Wschr.* 20 May, p. 289.
(9) SCHALM, O. W. & HARNIG, C. M. (1939). *J. Amer. vet. med. Ass.* **94**, 372.
(10) SWETT, W. W., MATHEWS, C. A. & GRAVES, R. R. (1939). *J. Dairy Sci.* **22**, 993.
(11) PETERSON, E. H. & HASTINGS, E. G. (1939). *Cornell Vet.* **29**, 11.
(12) BRYAN, C. S. (1938). *Vet. Med.* **33**, 72.
(13) BRYAN, C. S. & TAYLOR, G. E. (1938). *N. Amer. Vet.* **19**, No. 1, p. 26.
(14) JOHNSON, T. (1938). *J. comp. Path.* **51**, 69.
(15) WARD, A. H. (1938). *N.Z. J. Sci. Tech.* **20**, 109A.
(16) RÖNNEFAHRT, G. (1938). Inaug. Diss., Berlin.
(17) LITTLE, R. B. (1939). *Vet. Ext. Quart. Univ. Pa.* **39**, no. 74, p. 3.
(18) MEIGS, E. B., CONVERSE, H. T. B., BURKEY, L. A., ROGOSA, M. & SANDERS, G. P. (1938). *J. Dairy Sci.* **21**, 165.
(19) BURKEY, L. A., MEIGS, E. B., SANDERS, G. P. & ROGOSA, M. (1938). *J. Dairy Sci.* **21**, 124.
(20) STANG, LERCHE & NEIDER (1937). *Dtsch. tierärztl. Wschr.* **45**, 337.
(21) LUYKEN, P. (1938). Inaug. Diss., Berlin.
(22) MILLER, W. T. & JOHNSON, H. W. (1939). *Circ. U.S. Dep. Agric.* no. 514.
(23) UDALL, D. H. & JOHNSON, S. D. (1935). *Cornell Vet.* **25**, 57.
(24) MILLER, W. T. (1936). *Rep. N.Y. St. Ass. Dairy Insp.*, **10**, 221.
(25) PETERSON, E. H., HASTINGS, E. G. & HADLEY, F. B. (1938). *Cornell Vet.* **28**, 307.
(26) SHERMAN, J. M. & NIVEN, C. F. (1938). *J. infect. Dis.* **62**, 190.
(27) BENDIXEN, H. C. (1937). *Medlemsbl. danske Dyrlaegeforen.* **20**, 519, 539.
(28) LITTLE, R. B. (1938). *J. exp. Med.* **68**, 905.
(29) —— (1939). *Proc. Soc. exp. Biol., N.Y.*, **41**, 254.
(30) —— (1939). *J. Amer. vet. med. Ass.* **94**, 496.
(31) BROWN, J. H. (1937). *Cornell Vet.* **27**, 110.
(32) STEWART, D. F. (1938). *Aust. vet. J.* **14**, 180.
(33) DAVIS, J. G. & ROGERS, H. J. (1939). *J. Hyg., Camb.*, **39**, 446.
(34) MÉSZÁROS, I. (1936). *Közl. Oessyehas élét-és kortan Köréből*, **26**, 536.
(35) KLIMMER, M., HAUPT, H. & ROOTS, E. (1928). *Zbl. Bakt.* I Orig., **107**, 206.
(36) EDWARDS, S. J. (1938). *J. comp. Path.* **51**, 250.

(37) Plastridge, W. N., Anderson, E. O. & Seremet, J. S. (1938). *Bull. Storrs. agric. Exp. Sta.* no. 224.
(38) Davis, J. G., McClemont, J. & Rogers, H. J. (1939). *J. Dairy Res.* **10**, 59.
(39) Williams, W. L. (1937). *J. Dairy Sci.* **20**, 711.
(40) Davis, J. G. & Rogers, H. J. (1939). *J. comp. Path.* **52**, 132.
(41) Foot, A. S. & Shattock, P. M. F. (1938). *J. Dairy Res.* **9**, 166.
(42) Rowland, S. J. & Zein-el-Dine, M. (1938). *J. Dairy Res.* **9**, 182.
(43) —— —— (1939). *J. Dairy Res.* **9**, 174; **10**, 267.
(44) —— —— (1939). *J. Dairy Res.* **10**, 108.
(45) Johns, C. K. & Hastings, E. G. (1938). *Canad. J. Res.*, Sect. D, **16**, 6.
(46) Sanders, G. P. (1938). *J. Dairy Sci.* **21**, 153.
(47) Little, R. B. (1938). *Cornell Vet.* **28**, 23.
(48) Fay, A. C., Cave, H. W. & Atkeson, F. W. (1938). *Cornell Vet.* **28**, 40.
(49) Kretchmar, H. H. (1938). *J. Dep. Agric. W. Aust.* **15**, 281.
(50) Davis, J. G., McClemont, J. & Rogers, H. J. (1939). *J. Dairy Res.* **10**, 59.
(51) McClemont, J. & Davis, J. G. (1939). *J. Dairy Res.* **10**, 81.
(52) —— —— (1939). *J. Dairy Res.* **10**, 88.
(53) McCulloch, E. C. & Fuller, S. A. (1939). *J. Bact.* **38**, 447.
(54) Gwatkin, R., Hadwen, S. & Legard, H. M. (1937). *Canad. J. comp. Med.* **1**, no. 3, p. 7.
(55) Udall, D. H., Johnson, S. D. & Ferguson, J. (1938). *Vet. Rec.* **50**, 1417.
(56) Horwood, R. E. & Bryan, C. S. (1938). *Quart. Bull. Mich. agric. Exp. Sta.* **21**, 34.
(57) Brown, J. H. (1938). *J. Amer. med. Ass.* **111**, 310.
(58) Steck, W. (1938). *Schweiz. Arch. Tierheilk.* **80**, 181, 250; and *Control of Bovine Mastitis* (1939), Verlag: Paul Haupt.
(59) Kästli, P. (1937). *Schweiz. Arch. Tierheilk.* **79**, 168.
(60) Klimmer, M. & Haupt, H. (1938). *Arch. wiss. prakt. Tierheilk.* **73**, 139.
(61) Giebner, O. (1938). *Arch. wiss. prakt. Tierheilk.* **73**, 374.
(62) Steck, W. (1938). *Arch. wiss. prakt. Tierheilk.* **73**, 360.
(63) Seelemann, M. & Siemonsen, K. (1938). *Arch. wiss. prakt. Tierheilk.* **73**, 243.
(64) Stableforth, A. W. & Scorgie, N. J. (1938). *Vet. Rec.* **50**, 663.
(65) Gerstner, R. (1938). *Arch. wiss. prakt. Tierheilk.* **73**, 362.
(66) Gay, F. P., Clark, A. R., Street, J. A. & Miles, D. W. (1939). *J. exp. Med.* **69**, 607.
(67) Allott, A. J. (1937). *J. Amer. vet. med. Ass.* **91**, 588.
(68) Gildow, E. M., Fourt, D. L. & Shaw, A. O. (1938). *J. Dairy Sci.* **21**, 759.
(69) Miller, W. T., Mingle, C. K., Murdoch, F. H. & Heishman, J. O. (1939). *J. Amer. vet. med. Ass.* **94**, 161.
(70) Hoge, W. G., Halversen, W. V. & Cherrington, V. A. (1939). *J. infect. Dis.* **64**, 27.
(71) Little, R. B. (1939). *Cornell Vet.* **29**, 287.
(72) Bauer, H. & Gunderson, M. F. (1938). *J. Bact.* **36**, 567.
(73) Stableforth, A. W. (1939). *Vet. Rec.* **51**, 1243.
(74) Plastridge, W. N., Anderson, E. O., Williams, L. F. & Weirether, F. J. (1939). *Bull. Storrs. agric. Exp. Sta.* no. 231.
(75) Bendixen, H. C. & Minett, F. C. (1938). *J. Hyg., Camb.*, **38**, 374.
(76) Pennell, R. B. & Huddleson, I. F. (1937). *Bull. Mich. agric. Exp. Sta.* no. 156.
(77) Priestley, F. W. (1938). *Vet. Rec.* **50**, 137.
(78) Miles, A. A. (1939). *Brit. J. exp. Path.* **20**, 63.
(79) Miles, A. A. & Pirie, N. W. (1939). *Brit. J. exp. Path.* **20**, 83.
(80) Common, R. H. & Kerr, W. R. (1938). *Vet. Rec.* **50**, 727.
(81) Meyer, D. B. & Huddleson, I. F. (1938). *Cornell Vet.* **28**, 293.
(82) Scrivner, L. H. (1939). Thesis, Ithaca, N.Y.
(83) Ehrlich (1937). *Z. InfektKr. Haustiere*, **52**, 163.
(84) Hermann, G. (1937). *Berl. tierärztl. Wschr.*, 19 Nov., p. 715.

(85) Thumann, E. (1937). Inaug. Diss., Hanover.
(86) Rudolf, H. (1938). *Dtsch. tierärztl. Wschr.* **46**, 679.
(87) Seelemann, M. & Wolf, C. H. (1938). *Z. InfektKr. Haustiere*, **54**, 21.
(88) Glässer (1938). *Dtsch. tierärztl. Wschr.* **46**, 23.
(89) Klimmer, M. & Vogel, H. (1937). *Tierärztl. Mitt.* **18**, 4 and 23.
(90) Love, E. L. (1938). *Vet. Med.* **33**, 276.
(91) Elder, C. (1939). *J. Amer. vet. med. Ass.* **95**, 38.
(92) Timoney, J. F. (1938). *Vet. Rec.* **50**, 1235.
(93) Fitch, C. P., Bishop, L. M. & Boyd, W. L. (1937). *J. Amer. vet. med. Ass.* **91**, 22.
(94) Fröhle, O. (1938). Inaug. Diss., Munich.
(95) Seelemann, M. & Pfeffer, A. (1938). *Z. InfektKr. Haustiere*, **53**, 211.
(96) Meinicke, E. (1938). *Dtsch. tierärztl. Wschr.* **46**, 177 and 193.
(97) Hermann, G. (1939). *Berl. Münch. tierärztl. Wschr.* 1939, p. 158.
(98) Fleischhauer, G. & Canič, R. (1939). *Berl. Münch. tierärztl. Wschr.* p. 238.
(99) Fleischhauer, G. (1939). *Berl. Münch. tierärztl. Wschr.* p. 399.
(100) Sirk, F. (1937). *Wien tierärztl. Mschr.* **24**, 614.
(101) Plum, N. & Russeff, C. (1939). *Skand. VetTidskr.* **29**, 31.
(102) Grycz, E., Soltys, M., Tekliński, A. & Zylbertal, S. (1938). *Pam. pans. Inst. nauk. Gosp. Wiej. Pulawy*, no. 2, p. 37. (Suppl. to *Wiad. weteryn.* **18**, no. 223.)
(103) Duffau, E. (1939). *Ninth Congr. Fed. Soc. Sci. méd. Afr. du Nord.* **1**, 41.
(104) Bell, F. N. & Irwin, M. R. (1938). *J. infect. Dis.* **63**, 251.
(105) Irwin, M. R. & Bell, F. N. (1938). *J. infect. Dis.* **63**, 263.
(106) Ber, A. (1938). *Arch. int. Méd. exp.* **13**, 411, 427.
(107) Priestley, F. W. (1937). *Vet. Rec.* **49**, 1315.
—— (1938). *Vet. Rec.* **50**, 1793.
(108) Huddleson, I. F. (1935). *Rep. Div. Vet. Sci. Mich. St. Coll.*
—— (1936). *Rep. Mich. agric. Exp. Sta.*
(109) Fitch, C. P., Boyd, W. L. & Bishop, L. M. (1938). *J. Amer. vet. med. Ass.* **92**, 171.
(110) Fitch, C. P., Boyd, W. L., Bishop, L. M. & Kelly, M. (1939). *Cornell Vet.* **29**, 253.
(111) Wackerbarth, G. (1937). Inaug. Diss., Hanover.
(112) Fitch, C. P., Boyd, W. L. & Bishop, L. M. (1939). *Cornell Vet.* **29**, 338.
(113) Hoppe, R. (1938). *Wiad. weteryn.* **17**, 81.
(114) Heinen, A. (1938). Inaug. Diss., Berlin.
(115) Ariel, M. (1937). *Kpatologiceskoj morfolologu brucelleza u abortirovannykh. plodov. ovec.* Moscow: Viem. Publ. Dept.
(116) Jepson, A. & Jørgensen, J. (1938). *Medlemsbl. danske Dyrlaegeforen*, **21**, 49 and 79.
(117) Priestley, F. W. & McEwen, A. D. (1938). *J. comp. Path.* **51**, 282.
(118) Priestley, F. W. (1939). *Vet. Rec.* **51**, 1423.
(119) Scorgie, N. J. (1938). *J. Path. Bact.* **46**, 165.
(120) Pullinger, E. J. (1938). *J. Path. Bact.* **47**, 413.
(121) McEwen, A. D. (1940). *Vet. Rec.* **52**, 97.
(122) McEwen, A. D. & Priestley, F. W. (1938). *Vet. Rec.* **50**, 1097.
(123) McEwen, A. D. (1939). *J. comp. Path.* **52**, 129.
(124) McEwen, A. D., Priestley, F. W. & Paterson, J. D. (1939). *J. comp. Path.* **52**, 116.
(125) Haring, C. M. & Traum, J. (1937). *J. agric. Res.* **55**, 117.
(126) Haring, C. M. (1938). *J. Amer. med. Ass.* **92**, 52.
(127) Velu, H. & Zottner, G. (1938). *Maroc. méd.* **18**, 51.
(128) Dubois, C. (1938). *Bull. Acad. vet. Fr.* **11**, 383.
(129) Hardenburg, J. G. (1939). *J. Amer. vet. med. Ass.* **94**, 479.
(130) Thomsen, A. (1939). *J. comp. Path.* **52**, 192.
(131) Demnitz, A. (1938). *Dtsch. tierärztl. Wschr.* **46**, 122.
(132) Kerr, W. R. (1938). *Vet. Rec.* **50**, 717.

(133) Beach, B. A. (1939). *J. Amer. vet. med. Ass.* **94**, 374.
(134) Zeller, M. & Henninger, R. (1939). *Z. InfektKr. Haustiere*, **54**, 265.
(135) Kress, F. (1938). *Z. InfektKr. Haustiere*, **52**, 316.
(136) Wight, A. E. (1938). *J. Amer. vet. med. Ass.* **92**, 264.
(137) De Fosset, A. J. (1938). *J. Amer. vet. med. Ass.* **92**, 279.
(138) Anon (1938). *J. Amer. vet. med. Ass.* **92**, 301.
(139) Carroll, A. N. (1938). *J. Amer. vet. med. Ass.* **93**, 244.
(140) Anon (1939). *J. Amer. vet. med. Ass.* **94**, 325.
(141) McEwen, A. D. (1938). *Vet. Rec.* **50**, 699.
(142) —— (1938). *Vet. Rec.* **50**, 712.
(143) Anon (1938). *Norsk. Vet. Tidsskr.* **50**, 373.
(144) Holth, H. (1938). *Norsk. Vet. Tidsskr.* **50**, 158.
(145) Klemola, V. M. & Knuuttila, S. (1936). *Maataloust. Aikakausk*, **8**, 36.
(146) Evans, S. A. (1938). *Rep. Dep. vet. Sci., Tanganyika*, 1937, p. 23.
(147) Gilbert, S. J. (1937). *J. comp. Path.* **50**, 335.
(148) Guillermo, Lockhart, P. & Berninzoni, T. (1936). *Bol. mens. Direcc. Ganad. Montevideo*, **20**, 22.
(149) Rolle, M. (1937). *III Balti, Valsty. vet. Kongr. Protocol.* 1937, p. 48 (in German).
(150) Roots, E. (1937). *III Balti, Valsty. vet. Kongr. Protocol.* 1937 p. 23 (in German).
(151) Graub, E. (1937). *Schweiz. Arch. Tierheilk.* **79**, 20.
(152) Simič, T. V. & Duričić, M. (1936). *Jugoslav. Vet. Glasn.* **16**, 3.
(153) Itabashi, K., Ito, S., Watanabe, S., Tagima, Y. & Harako, K. (1938). *J. Jap. Soc. vet. Sci.* **17**, 66.
(154) Albiston, H. E. (1938). *Aust. vet. J.* **14**, 136.
(155) Hindmarsh, W. L. (1938). *Aust. vet. J.* **14**, 140.
(156) Alessandrini, A. (1938). *Ann. Igiene*, **48**, 205.
(157) Machado, A. (1938). *Bol. pec. Lisboa*, **6**, 15.
(158) Ivanoff, R. (1939). *Mon. Bull. agric. Sci.* **30**, 211 T.
(159) Topacio, T. & Acevedo, R. A. (1938). *Philipp. J. Anim. Indust.* **5**, 597.
(160) Anon (1938). *Arch. int. Brucellosis*, **1**, 33.
(161) Moore, W., Grinnelles, C. D. & Faulhaber, L. J. (1937). *Tech. Bull. N.C. agric. Exp. Sta.* no. 54.
(162) Martin, W. E. (1938). *J. Amer. vet. med. Ass.* **92**, 560.
(163) Hill, C. L. (1938). *J. Amer. vet. med. Ass.* **92**, 269.
(164) Parschau, J. (1937). Inaug. Diss., Berlin.
(165) Marienburg, H. (1938). *Dtsch. tierärztl. Wschr.* **46**, 228.
(166) Mazé, P. (1937). *C.R. Soc. Biol., Paris*, **126**, 740.
(167) Montgomerie, R. F. (1938). *Vet. Rec.* **50**, 380.
(168) Welch, H., Wentworth, J. A. & Mickle, F. L. (1938). *J. Amer. vet. med. Ass.* **111**, 226.
(169) Menefee, E. E. & Poston, M. A. (1939). *J. Bact.* **37**, 269.
(170) Miller, W. T., Mingle, C. K., Murdoch, F. M. & Heishman, J. O. (1939). *J. Amer. vet. med. Ass.* **94**, 161.
(171) Pallaske, G. (1938). *Berl. Munch. tierärztl. Wschr.* p. 752.
(172) Taylor, A. W. (1939). *J. comp. Path.* **52**, 140.
(173) Fitch, C. P. & Dodge, R. E. (1939). *Cornell Vet.* **29**, 29.
(174) Doyle, T. M. (1939). *J. comp. Path.* **52**, 89.
(175) Pavlov, P. (1938). *Réc. Méd. vét.* **114**, 790.
(176) Bosworth, T. J. (1937). *J. comp. Path.* **50**, 345.
(177) Fitch, C. P. & Bishop, L. M. (1938). *Cornell Vet.* **28**, 304.
(178) Sandholm, A. (1938). *Z. InfektKr. Haustiere*, **53**, 201.
(179) Cotton, W. E., Buck, J. M. & Smith, H. E. (1938). *Tech. Bull. U.S. Dep. Agric.* no. 629.

(180) Jamieson, S. R. (1938). *J. Path. Bact.* **47**, 353.
(181) Bond, E. W. (1938). *J. Amer. vet. med. Ass.* **92**, 453.
(182) Kliewe, H. & Schuppener, A. (1937). *Zbl. Bakt.* I Orig. **139**, 180.
(183) Wilson, G. S. & Schwabacher, H. (1937). *Tubercle, Lond.*, **18**, 161.
(184) Potter, T. S. (1937). *J. infect. Dis.* **60**, 88.
(185) Corper, H. J. & Cohn, M. L. (1938). *J. Bact.* **35**, 223.
(186) Bongert, J. G. (1938). *Dtsch. tierärztl. Wschr.* **46**, 579 and 599.
(187) Corper, H. J., Cohn, M. L., Simpson, M. G. & Bower, C. (1937). *Amer. Rev. Tuberc.* **36**, 763.
(188) Arloing, P., Thévenot, L. & Viallier, J. (1938). *C.R. Soc. Biol., Paris*, **129**, 582.
(189) Smithburn, K. C. (1937). *Amer. Rev. Tuberc.* **36**, 637.
(190) Chiti, G. (1938). *Zbl. Bakt.* I Orig. **142**, 313.
(191) Lockemann, G. (1938). *Zbl. Bakt.* I Orig. **142**, 79.
(192) Sher, B. C. & Sweany, H. C. (1939). *J. Bact.* **37**, 377; **38**, 411.
(193) Nikolić, N. (1938). *Zbl. Bakt.* I Orig. **141**, 66.
(194) Groh, E. (1938). *Zbl. Bakt.* I Orig. **142**, 190.
(195) Menzel, A. E. O. & Heidelberger, M. (1939). *J. biol. Chem.* **127**, 221.
(196) McCarter, J., Kanne, E. M. & Hastings, E. G. (1939). *J. Bact.* **37**, 461.
(197) Chiti, G. (1938). *Zbl. Bakt.* I Orig. **142**, 303.
(198) Suzuki, C. (1938). *Z. ImmunForsch.* **93**, 498.
(199) Boquet, A. (1937). *Rec. Méd. vét.* **113**, 696.
(200) Buxton, J. B. & Glover, R. E. (1939). *Sp. Rep. Ser. agric. Res. Coun. Lond.* no. 4.
(201) Celoria, M. (1938). *Boll. Sez. ital. Soc. int. Microbiol.* **10**, 69.
(202) Doig, A. T., Gemmell, G., Kayne, G. G., Linggood, F. V., Parish, H. J. & Westwater, J. S. (1938). *Brit. med. J.* 1, p. 992.
(203) Montgomerie, R. F. & Thomson, A. (1939). *Vet. Rec.* **51**, 229.
(204) —— —— (1938). *Vet. Rec.* **50**, 1759.
(205) Blount, W. P. (1938). *Vet. J.* **94**, 254.
(206) Papers read to the 13th Internat. Vet. Congress, 1938.
(207) Strube, G. (1938). Inaug. Diss., Berlin.
(208) Plum, N. (1938). *Acta path. microbiol. scand.* Suppl. no. 37, 438.
(209) Machens, M. (1938). *Berl. tierärztl. Wschr.* 13 May, p. 282.
(210) Rautmann, H. (1938). *Berl. tierärztl. Wschr.* 13 May, p. 279.
(211) Hermansson, K. A. (1938). *Skand. VetTidskr.* **28**, 553.
(212) Schultz, G. (1937). Inaug. Diss., Berlin.
(213) Wilkie, J., Edwards, S. J., Fowler, A. B. & Wright, N. C. (1937). *J. Dairy Res.* **8**, 311.
(214) Schumann, P. & Fritzsche, K. (1938). *Berl. tierärztl. Wschr.* 11 Feb., p. 77.
(215) Hemmert-Halswick (1938). *Dtsch. tierärztl. Wschr.* **46**, no. 2, p. 25.
(216) Hildenbrand, E. G. (1936). Inaug. Diss., Giessen.
(217) Kiedrowski, L. (1936). *Z. InfektKr. Haustiere*, **50**, 41.
(218) Fölger, A. F. (1938). *Acta path. microbiol. scand.* Suppl. no. 37, p. 179.
(219) Lange, B. (1937). *Z. Tuberk.* **77**, 249.
(220) Feldman, W. H. & Baggenstoss, A. H. (1938). *Amer. J. Path.* **14**, 473.
(221) Jansen, J. (1938). *Tijdschr. Diergeneesk.* **65**, 1218.
(222) Aubertin, E., Richard, J. & Perineau, G. (1938). *C.R. Soc. Biol., Paris*, **127**, 1101.
(223) Smithburn, K. C. (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 575.
(224) Kon, S. K. & Maddock, C. (1938). *Vet. Rec.* **50**, 1693.
(225) Schulte, W. (1937). Inaug. Diss., Hanover.
(226) Joyner, A. L. & Sabin, F. R. (1938). *J. exp. Med.* **68**, 325.
(227) Howe, J. S. (1939). *Proc. Soc. Exp. Biol., N.Y.*, **40**, 509.
(228) Benedetto, F. (1937). *Nuovo Ercol.* **42**, 367.

(229) Thijn, J. W. (1938). *Tijdschr. Diergeneesk*, **65**, 8.
(230) Plum, N. & Slynborg, N. C. (1938). *Maanedsskr. Dyrlaeg.* **50**, 473.
(231) Craig, J. F. & Davies, G. O. (1938). *Vet. Rec.* **50**, 1156.
(232) Stubbs, E. L. & Live, I. (1939). *J. Amer. vet. med. Ass.* **95**, 173.
(233) Wells, A. Q. (1938). *Brit. J. exp. Path.* **19**, 324.
(234) Griffith, A. S. (1939). *J. Hyg., Camb.*, **39**, 244.
(235) Griffith, A. S. & Pagel, W. (1939). *J. Hyg., Camb.*, **39**, 154.
(236) Saenz, A. & Canetti, G. (1938). *C.R. Soc. Biol., Paris*, **127**, 922.
(237) Negre, L. & Bretey, J. (1938). *Ann. Inst. Pasteur*, **61**, 109.
(238) Boer, H. D. (1936). *Versl. Tuberc. Stud. Comm. 'sGrav.* no. 10, p. 77.
(239) Goeters, W. (1936). *Klin. Wschr.* **15**, 45.
(240) Heymer, A. (1937). *Munch. med. Wschr.* **84**, 58.
(241) Lange, B. (1937). *Dtsch. med. Wschr.* **63**, 1465 and 1506.
(242) Oesterreich, E. (1937). *Beitr. Klin. Tuberk.* **89**, 128.
(243) Petragnani, G. (1937). *Bull. Off. int. Hyg. publ.* **29**, 1653.
(244) Rosa, A. (1937). *Riv. Pat. Clin. Tuberc.* **11**, 237.
(245) White, C. (1937). *Bull. Off. int. Hyg. publ.* **29**, 1649.
(246) Pikkarainen, J. (1938). *Art. sero-bakt. Inst. Univ. Helsinki*, **10**, 1.
(247) Morgan, M. T. (1937). *Bull. Off. int. Hyg. publ.* **29**, 1337.
(248) Rocher, H. L. & Laporte, R. (1938). *C.R. Soc. Biol., Paris*, **127**, 266.
(249) Saenz, A. (1938). *C.R. Soc. Biol., Paris*, **127**, 269.
(250) Weissfeiler, J., Morosowa, E. N. & Pesina, E. J. (1937). *Ann. Inst. Pasteur*, **59**, 259.
(251) Opie, E. L. & Freund, J. (1937). *J. exp. Med.* **66**, 761.
(252) Le Chiuton, F., Satrages, J., Berge, C., Pennaneac'h, J. & Dubreuil, J. (1937). *C.R. Soc. Biol., Paris*, **125**, 441.
(253) Griffith, A. S. & Glover, R. E. (1939). *J. comp. Path.* **52**, 57.
(254) Gräub, E. & Zschokke, W. (1938). *Schweiz. Arch. Tierheilk.* **80**, 289.
(255) Dessy, G. (1937). *Boll. Sez. ital. Soc. int. Microbiol.* **9**, 249.
(256) Buxton, J. B. & Glover, R. E. (1939). *J. comp. Path.* **52**, 47.
(257) Hensel, G. (1937). *Beit. Klin. Tuberk.* **90**, 387.
(258) Coulaud, E. (1938). *Ann. Inst. Pasteur.* **61**, 355.
(259) Lurie, M. B. (1939). *J. exp. Med.* **69**, 555.
(260) Arena, A. R. (1938). *C.R. Soc. Biol., Paris*, **127**, 557.
(261) Gottschall, R. & Bunny, W. E. (1938). *J. Immun.* **34**, 103.
(262) Seideman, R. M. (1939). *Amer. J. Hyg.*, Sect. B, **30**, 1.
(263) Bonnet, H., Thieffry, S. & Montefiori (1938). *C.R. Soc. Biol., Paris*, **128**, 585, 725.
(264) Plum, N. (1937). *Skand. VetTidskr.* **27**, 589.
(265) —— (1938). *Skand. VetTidskr.* **28**, 97.
(266) —— (1939). *Skand. VetTidskr.* **29**, 105.
(267) Stenius, R. (1938). *Vet. Rec.* **50**, 633.
(268) Feldman, W. H. (1939). *J. Amer. vet. med. Ass.* **95**, 152.
(269) —— (1938). *Avian Tuberculosis Infection.* The Williams and Wilkins Co., Baltimore, U.S.A.
(270) Timoney, J. F. (1939). *Vet. Rec.* **51**, 191 and 239.
(271) Griffith, A. S. (1938). *J. comp. Path.* **51**, 152.
(272) Russeff, C. (1938). *Dtsch. tierärztl. Wschr.* **46**, 135.
(273) Nielsen, F. W. & Plum, N. (1940). *Vet. J.* **96**, 6.
(274) Carmichael, J. (1938). *Vet. Rec.* **50**, 1138, 1147.
(275) Begley, P. T. (1938). *Vet. Rec.* **50**, 286.
(276) McKay, W. M. (1939). *Vet. Rec.* **51**, 782.
(277) Schmidt, H. W. (1938). *Dtsch. tierärztl. Wschr.* **46**, 482.

(278) Tobler, J. (1937). Inaug. Diss., Hanover.
(279) Smythe, R. H. (1939). *Vet. Rec.* **51**, 533.
(280) Blanchard, W. D. (1939). *Vet. Rec.* **51**, 236, 534.
(281) Hancock, R. C. G. (1939). *Vet. Rec.* **51**, 17.
(282) Pröscholdt (1938). *Prag. Tierärztl. Arch.* **18**, 85.
(283) Rolle, M. (1938). *Protokol. III Vet. Kongr. Balti. Staahen*, 1937, p. 33.
(284) Hungerford, T. G. (1939). *Aust. Vet. J.* **15**, 15.
(285) Cabot, D. A. E. (1938). *Vet. Rec.* **50**, 1233.
(286) Andersson, N. (1938). *Svensk. VetTidskr.* **43**, 3.
(287) Krantz, G. T. (1938). *Skand. VetTidskr.* **28**, 1.
(288) Hole, N. H. & Hulse, E. C. (1939). *J. comp. Path.* **52**, 201.
(289) Schmidt, J. & Messing, S. (1938). *Berl. tierärztl. Wschr.* **8**, April, 197.
(290) Ritter, H. & Nothdorff, G. (1938). *Zbl. Bakt.* I Orig. **141**, 171.
(291) Thomas, M. (1939). *C.R. Soc. Biol., Paris*, **130**, 512.
(292) Legrand, R., Gernez, C., Crampton, P. & Lefort, E. (1938). *C.R. Soc. Biol., Paris*, **128**, 204.
(293) Pallaske, G. (1939). *Arch. wiss. prakt. Tierheilk.* **74**, 99.
(294) Murphy, W. A. & Duerschner, D. R. (1938). *J. Lab. clin. Med.* **24**, 70.
(295) Guggenheim, A. & Finkelstein, M. (1939). *Amer. Rev. Tuberc.* **39**, 397.
(296) Glover, R. E. (1938). *Vet. Rec.* **50**, 981.
(297) (1938). *Rep. 13th Internat. Vet. Cong.* **1**, 399.
(298) Wight, A. E. (1938). *J. Amer. vet. med. Ass.* **92**, 444.
(299) Faulder, E. T. (1938). *J. Amer. vet. med. Ass.* **92**, 450.
(300) (1939). *Rep. Sp. Com. Tuberculosis, J. Amer. vet. med. Ass.* **94**, 323.
(301) Anon (1939). *J. Amer. vet. med. Ass.* **95**, 773.
(302) Rowlands, W. T. & Montgomerie, R. F. (1938). *Vet. Rec.* **50**, 1579.
(303) Kelland, J., Frood, J. L. & Doyle, T. M. (1938). *Vet. J.* **94**, 93.
(304) Allan, H. B. (1938). *Vet. Rec.* **50**, 119.
(305) Stenius, R. (1937). *Suom. Elainlaakaril.* **43**, 6.
(306) Nielsen, F. W. (1938). *Vet. J.* **94**, 117.
(307) —— (1938). *Dtsch. tierärztl. Wschr.* **46**, 513.
(308) Weisstanner, M. (1937). Inaug. Diss., Berlin.
(309) Pröscholdt (1938). *Prag. tierärztl. Arch.* **18**, 85.
(310) Venema, H. (1938). *Tijdschr. Diergeneesk.* **65**, 1209.
(311) Beijers, J. A. (1938). *Tijdschr. Diergeneesk.* **65**, 1213.
(312) Doeve, W. C. A. (1938). *Ned.-ind. Bl. Diergeneesk*, **50**, 45.
(313) Debelić, S. (1938). *Vet. Arhiv.* **8**, 460.
(314) Lucas, A. P. (1938). *Bol. pec. Lisboa.* **6**, 23.
(315) Anon (1938). *Bol. pec. Lisboa.* **6**, 51.
(316) Ivanoff, K. (1939). *Mon. Bull. agric. sci.* **30**, 211 T.
(317) Rolle, M. (1938). *Protokol. III Vet. Kong. Balti. Staaten*, 1937, p. 33.
(318) Holmes, M. J. (1937). *Med. J. Aust.* **2**, 813.
(319) —— (1937). *Aust. vet. J.* **13**, 6.
(320) Ferauge, A. (1938). *Ann. Méd. vét.* **83**, 17.

(*MS. received for publication* 29 *July* 1940.)

CAMBRIDGE: PRINTED BY W. LEWIS, M.A., AT THE UNIVERSITY PRESS